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From the Nobel Institute for Neurophysiology, Stockholm 60.

Generalized Inhibition of the Retinal Ganglion Cells in Relation to Wave-Length and State of Adaptation.

By

EBERHARD DODT.[1]

Received 21 December 1955.

While studying the spectral distribution of sensitivity of individual retinal elements in the darkadapted rabbit's eye it was observed that some elements which responded strongly to orange light around 6,000 Å did not respond at all to green light around the visual purple optimum of 5,000 Å. In this work suprathreshold energy was used together with a flickering spectral stimulus as the constant index, not the absolute threshold as in most previous experiments from this laboratory, so that it was possible to vary the energy upwards and downwards within a considerable margin. It turned out that the silence of the element stimulated with green light (to which all elements are particularly sensitive) was an inhibition due to overstimulation, something reminiscent of glare in vision. As soon as stimulus intensity had been lowered, the element again began to respond to flicker at wave-length 5,000 Å. Return of the response could also be produced by light adaptation.

Tests with other elements and rabbits soon made it clear that this total inhibition of the flicker response in dark adaptation was fairly common and that it also could be demonstrated in every wave-length. Up to then the experiments had been concerned with determining the energy necessary in each wave-length for flicker-

[1] Fellow of the Rockefeller Foundation. Present address: William G. Kerckhoff Institute, Bad Nauheim, Germany.

fusion to light intermittent at a slow constant rate, *i. e.* to determine the spectral sensitivity distribution by the flicker method as applied to individual ganglion cells. Clearly, however, in view of the inhibition to overstimulation, it would now also be possible to determine a second similar spectral distribution curve at a higher level of intensity. As stimulus energy in any given wave-length gradually was increased, the element would first show flicker-fusion, then begin to flicker violently, finally, on further increase of stimulus strength, inhibition would set in so that ultimately a second limiting value, the fusion frequency at which inhibition overtook excitation, was obtained. Thus arose the interesting question of whether the two spectral distributions of sensitivity, the low-energy curve for excitation and the high-energy curve for inhibition, would be identical. All graphs in this paper are based on reciprocals of the quantum intensity necessary for flicker-fusion as constant index.

Methods.

Rabbits injected with urethane subcutaneously (1.5 g/kg) were given Flaxedil (5—10 mg/kg) for immobilization and put under artificial respiration. The animal was placed in the head holder, one eye was opened and a microelectrode inserted; then the eye was left to dark-adapt for a few hours before individual ganglion cells were isolated for experimentation in the manner of this laboratory (GRANIT 1947).

The Hilger-Tutton monochromator, used here in some previous work, was recalibrated for the present experiments, this time with a 20 % solution of $CuSO_4$ in a trough 1 cm in width in the light beam in order to prevent stray light from the red end interfering with the measurements in the blue end of the spectrum. In previous experiments the Wright monochromator which gives greater purity had been used for such work but, since the intention was to study suprathreshold values, more energy was needed than is obtainable in the Wright instrument. As before, a Hilger-Schwarz vacuum thermopile, Model FT 12 in conjunction with a Siemens Super-galvanometer was employed in the spectral energy measurements. The deflexion of the galvanometer was optically magnified in a ratio of 1 : 15. All filters and wedges used for varying light intensity were recalibrated in the visible spectrum and, for the wedges, curves to serve in the final energy calculations for gradient density in each wave-length were plotted.

The procedure was to begin measurements in every wave-length by determining the thresholds for inhibition and excitation in terms of flicker-fusion, then to make a run through the spectrum between 4,200 and 5,600 Å at intervals of 100 Å and between 5,600 and 6,000 Å at intervals of 200 Å. Between every wave-length tested a control of the general sensitivity level was taken in terms of both inhibition and

excitation using 5,000 Å as the reference standard. When one run was completed, another followed so that, in the end, up to 250 individual measurements were available for the calculation of the spectral sensitivity distributions.

Some measurements were also made of the relation between flicker-fusion and log energy (often referred to as the Ferry-Porter curve) but in this case there was, of course, also a second curve to be measured which represented the fusion point as determined by inhibition.

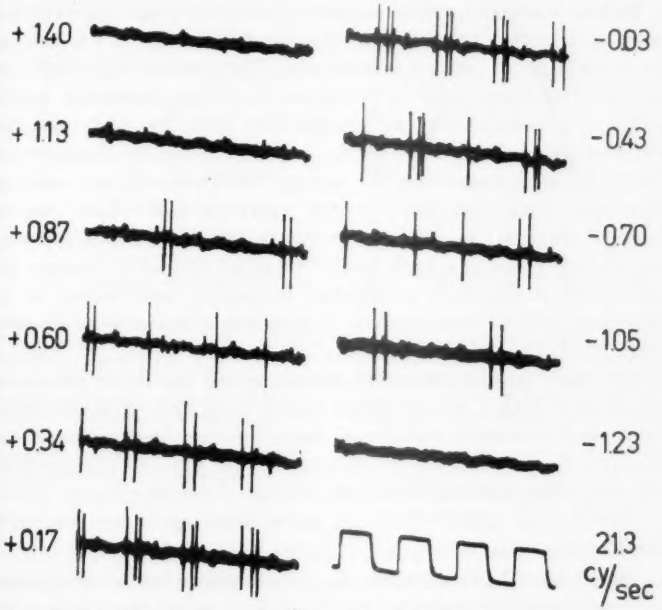

Fig. 1. Rabbit (Flaxedil, urethane); 3 hrs dark adaptation. Microelectrode records from single ganglion cells responding to intermittent light at rate 21.3 flashes/sec at the relative energies indicated alongside records in log units. Wave-length 5,300 Å.

Results.

1. *The generalized inhibition.* Fig. 1 demonstrates the two thresholds for flicker-fusion at a rate of 21.3/sec of intermittent stimulation. Log energy values are given alongside each record. Beginning at threshold intensity -1.23 the eye required energy level -0.43 for regular flicker. The excitatory fusion point was at -0.70 and it is defined as the intensity at which the spikes fail to follow the rhythm of the flashes. As energy increased

from this level, flicker with three spikes per flash was gradually supplanted by flicker with two spikes per flash and at + 1.13 there was complete inhibition. Fusion again took place somewhere between + 0.60 and + 0.87 and this is the inhibitory fusion point. With either fusion point as constant index it is possible to plot spectral distribution curves of sensitivity, as mentioned in the introduction.

Before analyzing colour sensitivity some general observations were made on the phenomenon of inhibition. In Fig. 2 it is studied as a function of rate of stimulation. The flicker frequency was set on the instrument (a Velodyne feedback-controlled motor rotating a sectored disk as described by ENROTH 1952) and the corresponding energy values for critical flicker or fusion were found by adjustments on the wedge. The lower curve is nothing but the well-known Ferry-Porter curve (a fact which can be better visualized by tilting the figure 180° in an anticlockwise direction). There is a kink in the curve at around 12 flashes per sec which is generally interpreted to signify that "cones" or at any rate a faster type of new element begin to make themselves felt. It can be seen with critical flicker also in the cat's retina (DODT and ENROTH 1953). The upper curve in the figure illustrates the region within which fusion owing to inhibition takes place.

With frequencies below the value of the 'kink' it was not possible to demonstrate inhibition; nor did inhibition occur with single flashes. Intermittent light stimuli above the energy values included in the graph elicited an initial discharge before inhibition developed.

Fig. 2 also shows that the energy necessary for the occurrence of inhibition, as measured by the fusion point, depended upon flicker frequency. In this regard individual ganglion cells differed a great deal. Some could not be inhibited at all, whatever the amount of energy used, others gave the inhibitory response at energy levels 10—100 times lower than the usual values (at the frequency illustrated in Fig. 1). The cells which could not be completely inhibited nevertheless showed some signs of inhibition in that for some seconds after the first flash they failed to follow the stimulus rhythm. This effect, however, was transient (cf. DODT and HECK 1954).

The generalized inhibition of the flicker response was seen also in decerebrate cats and thus did not require centres above the retinal level.

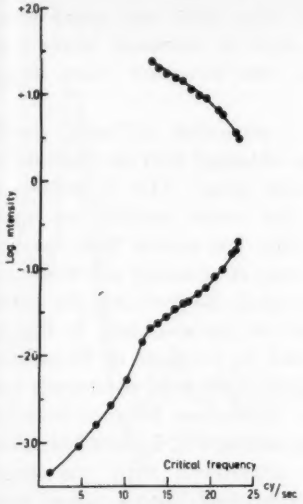

Fig. 2. Rabbit (Flaxedil, urethane); 3 hrs dark adaptation. Log stimulus intensity (wave-length 5,300 Å) necessary for 'excitatory' (below) and 'inhibitory' fusion (above) at different rates of intermittent stimulation. The two curves recorded in parallel by alternating between the lower and upper value for fusion or so-called critical flicker.

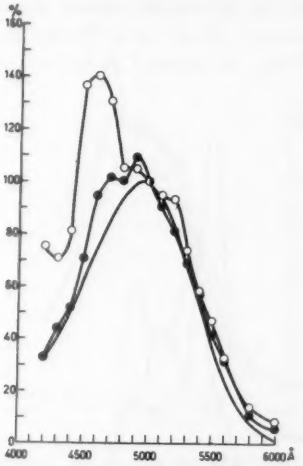

Fig. 3. Average spectral distributions of 10 elements as determined by flicker-fusion as constant index in darkadapted rabbits. Spectrum of equal quantum intensity; values in terms of 5,000 Å at 100 per cent. *Filled circles*, 'excitatory' fusion point; *open circles*, 'inhibitory' fusion point, as explained in text. Curve not based on observations represents the type of visual purple known as the pigment 497 of Dartnall.

2. *Spectral distributions for excitation and inhibition.* In Fig. 3 the curve drawn in full (independent of the observations) shows DARTNALL's (1953) visual pigment 497 which is likely to represent the visual purple of the rabbit's retina. Comparisons with the absolute threshold of individual elements have been made by DODT and ELENIUS (1955). When now flicker fusion was used (the curve drawn between filled circles) at frequencies of stimulation corresponding to the upper portion of the graph of Fig. 2, the result was a spectral distribution of sensitivity that agreed with the values obtained by DODT and ELENIUS with threshold as constant index. In both cases, and as shown in the figure for flicker only, there were considerable differences between Dartnall's curve for the homogeneous visual purple pigment 497 and the physiological result, particularly from 4,600 to 4,800 Å. Clearly some other substance determined the high sensitivity to

bluish light. Specific sensitivity to blue light was noted even at the threshold which required about a thousand times less energy (DODT and ELENIUS) than was necessary when using flicker at around 20/sec as index.

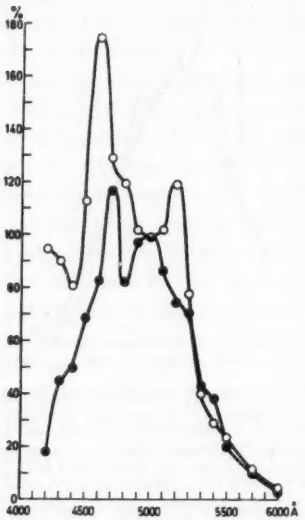

Fig. 4. As Fig. 3, but for a single element.

A somewhat different result was obtained with the 'inhibitory fusion point'. This is presented in the curve marked by open circles. The narrow 'blue' band is greatly emphasized and there are humps in the green and the violet parts of the spectrum. In Fig. 3, based on averages of 10 curves, minor shifts tend to smooth out the differences between individual results. Fig. 4, therefore, adds an experiment with one single ganglion cell. The retina had been darkadapted for 4 hours and then, to threshold flicker-fusion as index, responded with the curve drawn between filled circles. The 'inhibitory fusion' gave the curve between open circles. There was not enough energy available in the spectrum to complete analysis of what now appears as a small rise at 4,200 Å. Similarly in DODT and ELENIUS' work the rise in the blue was found shifted more towards the short wave-lengths the greater the hump. In Fig. 4 there is also a very definite top at 5,200 Å.

In Fig. 5, the values for the excitatory or threshold fusion (filled circles) and for visual pigment 497 (open circles) have been subtracted from the inhibitory fusion values. Based on the averages of Fig. 3, these two curves then show the deviations of the high-energy curve for inhibition. The differences in the short wave-lengths are far beyond any limits of error. The rise in 5,200 Å also seems significant. It was very common in the individual experiments as well as in those of DODT and ELENIUS (1955). The rise in 5,550 Å is perhaps less definite.

3. *Effect of state of adaptation.* Complete and lasting inhibition was only seen in the eyes that had been darkadapted for over

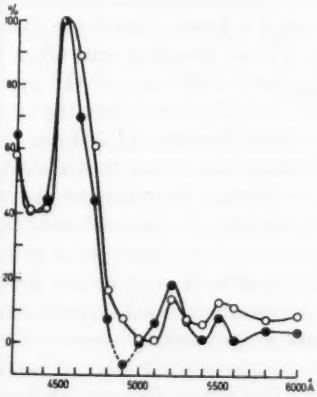

Fig. 5. Deviations of the 'inhibitory' curve of Fig. 3 from the threshold distribution of the spectral sensitivity (*filled circles*) and from the curve for pigment 497 (*open circles*), based on maximum given as 100 per cent.

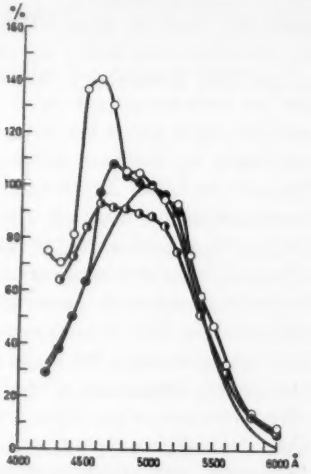

Fig. 6. Comparisons between the 'inhibitory' curve's spectral distribution of sensitivity (*open circles*) and the same functions for absolute threshold as index in dark adaptation (*filled circles*) and light adaptation (*half-filled circles*) as well as for visual pigment 497.

$1^1/_2$ hour. This time is approximately the one found by RUSHTON and his collaborators (1955) for full dark adaptation in rabbits. By direct measurements of the visual purple concentration in this eye with the aid of Rushton's densitometer they found light adaptation to be followed by 80 min of regeneration of visual purple as compared with only 30—40 min in man.

With shorter times in the dark the effect of dark adaptation was noticeable in a lengthening of the time necessary before the steady response to intermittent illumination changed into a flickering response. The same type of behaviour was found when, in an eye with well developed inhibition, selective adaptation to monochromatic light at maximum strength of the monochromator was allowed to proceed for several minutes. Systematic studies of the effects of selective adaptation on the generalized inhibition have not been made but it is clear from the observation protocols, in which the control wave-length 5,000 Å has been followed throughout every experiment, that blue-green light increased the inhibitory threshold more than orange and red light.

Discussion.

Complete generalized inhibition of the kind described above has not been recognized before though it is known that in the dark-adapted cat's retina the discharge of some ganglion cells often is depressed by stronger stimuli. Relative inhibition of the eye's capacity to follow intermittent light is well-known since the electroretinographic work of GRANIT and RIDDELL (1934) on the frog's eye, showing that it lasted some time before a well dark-adapted frog's eye was capable of following intermittent stimulation to strong light. DODT and HECK (1954), with frogs and cats, showed how this non-flickering phase of the response to intermittent light depended upon duration of dark adaptation. While the fusion frequency of retinal elements in darkadapted cats often decreases when light intensity is increased (ENROTH 1952), there is, in lightadapted cats, merely a general increase of fusion frequency with stimulus intensity (DODT and ENROTH 1953).

In vision it was shown long ago by SCHATERNIKOW (1902) and confirmed by LYTHGOE and TANSLEY (1929) and ENROTH and WERNER (1936) that the perceived fusion frequency decreases in dark adaptation. Totally colour blind, tested electroretinographically, may with strong stimuli refuse to respond intermittently to intermittent light (DODT and WADENSTEN 1954; WADENSTEN 1955).

Another interesting new finding in this work is that at this boundary of rivalry between excitation and inhibition also the spectral distribution of sensitivity of the elements concerned plays a rôle. The curves obtained differ from both scotopic and photopic sensitivity distributions obtained from threshold measurements (DODT and ELENIUS 1955), as shown in Fig. 6. This suggests interaction with consequent shifts of emphasis from one type of photoreceptor to another. Since dark adaptation is necessary, the over-stimulated rods somehow must inhibit cones, as suggested by GRANIT and RIDDELL (1934) and DODT and HECK (1954) but apparently this process does not suppress all cones equally. The reasons for the differentiation, so obvious in the curves presented, is at the moment unknown.

The results also would seem to be of some general interest from the point of view of 'glare', which, in sudden shifts from darkness to a considerably higher level of illumination, is so deleterious to vision. The present results suggest that blue light would be partic-

ularly apt to cause glare and this, in point of fact, has been reported by SCHOUTEN (1934). SCHOBER (1954) has ascribed this effect in the darkadapted eye to the greater amount of short wavelengths in the irradiated light.

Summary.

Complete generalized inhibition of individual retinal elements was observed in response to flickering light of increasing intensity in the darkadapted rabbit's eye. As soon as stimulus intensity had been lowered, the elements again began to respond to flicker.

The inhibition was confined to frequencies of intermittent light above the 'kink' in the Ferry-Porter curve. Within the upper part of this curve the energy required for inhibition gradually decreased with increase of flicker frequency.

Lasting inhibition was only seen in the eyes that had been darkadapted for over $1^1/_2$ hour. With shorter times of dark adaptation there was only a transient non-flickering phase of the response to intermittent light depending upon duration of dark adaptation and flicker frequency. Immediate return of the flickering response was produced by light adaptation.

Simultaneous determinations of energy necessary in each wave-length for the excitatory or threshold fusion on one hand and the inhibitory fusion point on the other reveals two different sensitivity curves. Whereas the low-energy curve for excitation practically agrees with the spectral distribution of sensitivity at the absolute threshold, the high-energy curve for inhibition shows greatly increased sensitivity to blue light and humps occur in the green and the violet parts of the spectrum.

The results would seem to be of some general interest also from the point of view of 'glare'.

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From the Institute of Physiology, University of Uppsala, Sweden.

Water Permeability of the Isolated Stomach of the Mouse.

By

K. J. ÖBRINK.

Received 27 December 1955.

The increasing interest in permeability problems in biological systems and several new approaches in the study of transport of matter through membranes have thrown new light upon the mechanism of gastric acid secretion. It has been clearly demonstrated that the concentration of hydrogen ions in the gastric juice is much higher than could be effected by a Donnan distribution (CONWAY and BRADY 1948, HOLLANDER 1943, TEORELL 1933). Likewise it has been proven that the energy demand for its formation is much higher than could be accounted for by the possible tendency to transudation due to the blood pressure (DAVIES and TERNER 1948, 1949). The only possible explanation for the accumulation of the hydrogen ions is some kind of an "active transport" *i. e.* a transport that depends on the expenditure of energy from other sources than the hydrogen ions themselves.

Theories for such an energy transformation have been presented by many authors (for reviews see CONWAY 1953, DAVIES 1951 and HEINZ and ÖBRINK 1954). Mostly these theories consider the anions as passively transported but HOGBEN (1951, 1952) and REHM, HOKIN, DE GRAFFENREID II, BAJANDAS and COY (1951) have suggested that also chloride is actively transported and creates the existing electrical potential across the mucosa (also cf. HEINZ, ÖBRINK and ULFENDAHL 1954). According to REHM (1950) and REHM and ENELOW (1945) this potential drop may have a controlling function in the secretion of the HCl.

Quantitative studies of energy demand, efficiency and enzymatic interactions have revealed some fundamental results: 1) The secretion is absolutely dependent on oxygen supply (glycolysis plays only a minor part (DAVENPORT and CHAVRÉ 1952). 2) The energy is probably transmitted through energy rich phosphates (CONWAY 1953 and DAVIES 1951). 3) The source of the hydrogen ions cannot be an organic precursor (CONWAY 1953) but must be the plasma water (DAVIES 1951).

Although many of the proposed mechanisms would be essentially possible there are important gaps in our knowledge about the secretion process. For instance: Why does histamine act as a stimulus for the secretion, and why does the secretion stop between the meals? That it does not act through changes in the circulation seems to be proven by (unpublished) experiments by Nordgren in this laboratory.

An explanation could be that histamine either takes part as a link in the H atom transport system (CRANE, DAVIES and LONGMUIR 1948) or that it controls the water output. The last mentioned possibility seems to be supported by experiments by LINDE, TEORELL and ÖBRINK (1947). They instilled isotonic glycine in Heidenhain pouches in dogs and found a close correlation between the histamine dose and volume secretion, while the output of acid changed independently of the two. A possibility is therefore that histamine controls the water permeation through the secreting cells. Thus a knowledge of the water permittivity of the secreting cells would be valuable, but it seems rather difficult to differentiate between possibly existing differences in different cells. It should no doubt be of fundamental interest to test whether the parietal cells are much more permeable to water than are the surface epithelial cells (SEC), as is one of the basic assumptions in the theory of REHM, SCHLESINGER and DENNIS (1953). Furthermore it ought to be shown whether histamine changes the permeability to water or not.

The first step in solving these problems is of course to determine the over all permittivity of the mucosa irrespective of its different structures. Actually REHM et al. made their conclusions about the low permeability of water through the SEC by such over all permeability studies (REHM, SCHLESINGER and DENNIS 1953). This average permeability to water is of an equally great importance for evaluating the fact that the mucosa is permeable to all the different ions in the primary secretion (H^+, Cl^- and K^+)

(TEORELL 1933, 1939, COPE, COHN and BRENIZER 1943, ÖBRINK 1948, TERNER 1948, 1949, a, b, CRANE and DAVIES 1949, LINDE and ÖBRINK 1950, FRIEDMAN 1951, 1952). In no permeability studies on the gastric mucosa has any consideration been given to possible movement of water. Thus the permeability coefficients for the ionic components of the gastric juice may have been incorrectly determined.

According to TEORELL (1933, 1947) the acidity regulation is to a great extent a diffusion-like process. The hydrochloric acid is thought to be secreted in a practically plasmaisoosmotic or slightly hypertonic concentration, but after being expelled into the lumen the hydrogen ions diffuse back to the blood due to the concentration gradient whereas the sodium ions for the same reason move in the opposite direction (the potassium ions are from the beginning very close to equilibrium). The difference in the ionic mobilities is electrically balanced by a net flux of chloride ions from the lumen to the blood. This makes the juice slightly hypotonic, which has also been demonstrated by GILMAN and COWGILL (1931, 1933) and LIFSON, VARCO and VISSCHER (1941, 1943). Of course this hypotonicity is counterbalanced by the tendency of water to diffuse in the direction of the higher "osmotic" pressure (lower activity) and the net result will depend on the mutual net fluxes of ions and water. As the permeability coefficients of the participating ions (H^+, Na^+ and Cl^-) are determined neglecting any net water fluxes, it is of importance to determine this net flux of water before an eventual evaluation of the true permeability coefficients of the ions can be made.

Under physiological conditions a large net transport of water takes place after stimulation. The magnitude of this net flux is dependent on the degree of stimulation and its maximum is found in dog and cat to be about 0.01 ml min^{-1} cm^{-2}. As the scope of this paper is the determination of the water permeability through the gastric mucosa, it is obvious that a large net transport of water makes it difficult to determine a diffusion permeability coefficient. Therefore a method used by DAVENPORT (1951) was adopted. It involves the use of isolated stomachs from frogs or mice, filled with a solution to a pressure that counterbalances the secretion pressure such as to keep the net transport of water at practically zero.

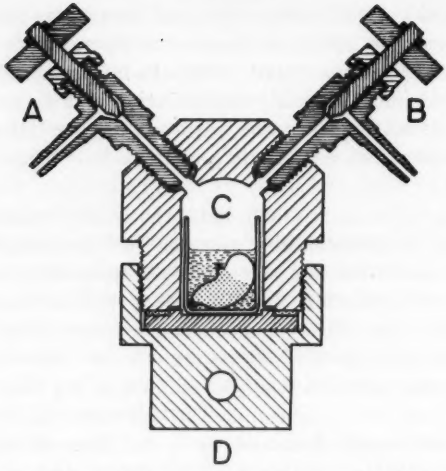

Fig. 1. Steel bombs in which the mouse stomachs were incubated in a water-bath (37° C) and 4.2 atm. oxygen pressure.

Methods.

Stomachs from frogs (Rana temporaria) and white mice were used. The animals were decapitated and bled out and the stomachs excised. In the case of frogs the mucosa was isolated from the rest of the stomach but the stomachs from mice were used as a whole.

The stomachs were filled with Krebs solution (see DAVENPORT 1951) to a pressure of 75 cm H_2O (mice) or 25 cm H_2O (frogs).

The filled stomachs were then immersed in 5 ml Krebs solution containing isotopic water, and brought into a pure oxygen atmosphere. The frog stomachs were brought into a Warburg apparatus while the bottle with the mice stomachs were exposed to an oxygen pressure of 4.2 atm. in a steel-bomb shown in fig. 1. (The method is exactly adopted after DAVENPORT 1951 and for a more detailed description the reader is referred to his papers.) To some of the outside solutions 20—40 μg/ml of histamine-dihydrochloride (frogs) or 1 μg/ml of carbacholine-chloride (mice) were added as stimulants for the acid secretion. After incubation in the oxygen atmosphere at 37° C for different lengths of time (10—120 minutes) the stomachs were taken out, weighed and their contents as well as that of the outside solution was analyzed for acidity and isotopic water.

Isotopic water analyses.

Two different kinds of isotopic water was used, namely D_2O and H_2O^{18}. D_2O was obtained from Norsk Hydro, Rjukan, Norway at 99.77 % (w/w) concentration (99.74 mol % D in water) and H_2O^{18} from Harwell, England in a concentration of 9.37 mol % O^{18}.

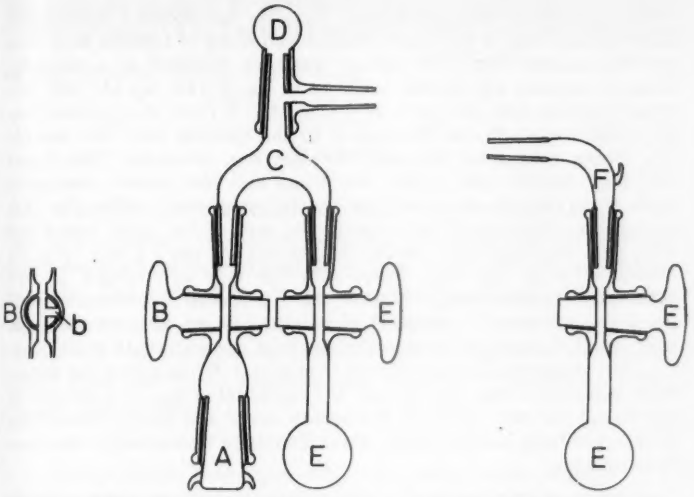

Fig. 2. *Equilibration unit* in which the isotopic waters HDO or H_2O^{18} were equilibrated with H_2 or CO_2. For description of its use see text.

Usually D_2O was added to the Krebs solution to make a final concentration below 6 mol % D in water (the dimension "mol % D in water" will henceforth be shortened to just "mol % D") which implies that less than 0.3 mol % D or less than $^1/_{20}$ was in the form of D_2O and the rest as HDO. When in the following the concentration is expressed as "mol % D" it means that the actual concentration of tagged water (in mol %) is almost double that figure. (Mol % D in water = $D_2O + {}^1/_2$ HDO when the water concentrations are expressed as $100 \times$ mol-fraction = mol %).

H_2O^{18} was added to a final concentration of about 0.075 mol % O^{18}.

The analysis of deuterium followed a principle that was first suggested by FARKAS (1936). The water to be analyzed was equilibrated with hydrogen gas in the presence of a platinum catalyst. The resulting ratio HD/H_2 in the gas phase was determined by a mass spectrometer. For details see KIRSCHENBAUM (1951, p. 205 ff). O^{18} was analyzed in a similar way only equilibrated with CO_2^{16} and determined from the ratio $CO^{16}O^{18}/CO^{16}O^{16}$. (This latter method was suggested by COHN and UREY 1938.)

Details of the procedure:

a) Water purification is not of extreme importance in the equilibration method but without a preceding distillation the platinum catalyst was poisoned. Therefore 1 ml of the solution was completely distilled in the presence of $KMnO_4$ and Na_2O_2.

b) Equilibration with H_2: To the distilled water was added about 5 mg of PtO_2 as a catalyst and 3 mg of fumaric acid as a peptizing agent. The water sample was then attached to a specially designed vacuum set-up that is shown in fig. 2. The cup (A) with the water together with the parts B + C + D + E form the equilibration unit, which was attached through D to the vacuum line. The sample was frozen with solid CO_2 and the unit was evacuated. The water was then thawed and frozen two times and the system evacuated each time, which outgassed the water completely. After the last evacuation stopcock E was closed, the rest of the unit was filled with pure tank hydrogen, stopcock B closed and parts C and D again evacuated (0.04 mm Hg). Note: Also the bore of stopcock B was evacuated simultaneously because of its special construction (see fig. 2, the left part). — Stopcock D was then closed, E opened and the unit detached from the vacuum line and kept in a waterbath at 25° C ± 0.05, for more than 5 hours (usually over night). While still in the waterbath stopcock B was opened and the equilibrated gas in A let out in the rest of the unit. Stopcock E was now closed and the gas-containing E detached from the rest of the unit and through F attached to the mass spectrometer.

c) The mass spectrometer used was a Consolidated-Nier Model 21—201 60° instrument. In the low mass region it could not be used for direct ratio measurements but had to be used on an absolute scale. The masses 3 and 2 were focused by voltage scanning.

d) Equilibration with CO_2: After the first equilibration a new part E was attached to the equilibration unit with the same water sample in A. The water was again outgassed but the unit this time filled with CO_2. (The presence of platinum oxide and fumaric acid does not do any harm.) Again a similar equilibration at 25° C followed and a similar analysis in the mass spectrometer of masses 46 and 44. This time a direct reading of the ratio M 46/M 44 was possible which yielded very accurate results.

Calculations:

Mol % D in water. The ratio mass 3/mass 2 was corrected for the presence of H_3^+ before being used for estimation of the mol % D. The formula derived by FISHER and POTTER (1944) (quoted from KIRSCHENBAUM 1951) was used:

$$\text{Mol \% D in water} = \frac{396\frac{[HD]}{[H_2]} + 194}{3.87 + 3.96\frac{[HD]}{[H_2]} + \frac{[H_2]}{[HD]}}$$

Mol % O^{18} was calculated from the ratio mass 46/mass 44. That ratio had first to be corrected by a factor due to the impossibility of a perfect focusing of the two masses on the two detectors and due to

the presence of mass 45 ($C^{13}O^{16}O^{16}$). Experimentally this correction factor was found to be 1.061. The corrected ratio R was then introduced in the formula: Mol % O^{18} in water $= \frac{100\,R}{2.088 + R}$

Results.

It must first be concluded that many of the experiments with the frog mucosae showed a too rapid equilibration of isotopic water between inside and outside to make any accurate calculation possible. The method has to be somewhat modified before these rapid fluxes can be measured. Any further discussion of these results will therefore be omitted. In the following only the experiments from the mouse stomachs will be reported; in table I these results are collected.

Table I.

Average figures from experiments with isolated mouse stomachs.

	M	s(M)	n
Weight of the filled stomach (g)	2.66	—	60
	1.58*	—	21
Change in volume ($\triangle$w) (g)	+ 0.007	± 0.004	60
	— 0.008*	± 0.004	21
k_D (cm sec^{-1})	$1.49 \cdot 10^{-5}$	$\pm\ 0.08 \cdot 10^{-5}$	43
$k_{O^{18}}$ (cm sec^{-1})	$1.91 \cdot 10^{-5}$	$\pm\ 0.09 \cdot 10^{-5}$	25
$k_{O^{18}}/k_D$	1.29	± 0.07	17

* results obtained by Davenport.
$k_D = k_{HDO}$; $k_{O^{18}} = k_{H_2O^{18}}$.

M = mean.
s(M) = standard error of the mean.
n = number of experiments.

Volume changes.

In the introduction it was pointed out the desirability of avoiding any net transport of water, which was measured by weighing the stomachs at the start and end of the experiment. This procedure necessarily included an error and small increments or decrements could not be avoided, but in 60 experiments with the average weight = 2.66 g the average change ($\triangle$w) was + 0.007 g ± 0.004 g, *i. e.* not significantly different from zero. The same result was obtained from figures put at my disposal by Dr. DAVENPORT, where 21 experiments gave a $\triangle$w of — 0.008 ± 0.004 g. Thus it seemed rather safe to neglect any net transport as being of importance. For an extra check see below.

The permeability coefficient.

The concentrations outside and inside the stomach were determined at time *t*. BOCHSLER (1948) suggested that if a suitable dimension is used for the water escaping tendency it obeys the simple diffusion law of Fick, $\frac{dn}{dt} = -DA \cdot \frac{dc}{dx}$. This is probably not true unless the water molecules move independently of each other. In the case of tracer experiments this is certainly what happens and Fick's law can be used. The escaping tendency could most conveniently be expressed in mol-fraction or mol % (according to the law of Raoult). The isotopic water abundance was therefore expressed as "mol % D in water" or "mol % O^{18} in water", *i. e.* the denominator in the mol fraction accounts only for the two water components and is not corrected for the dissolved ions. In the case of D_2O or H_2O^{18} in trace amounts this correction can be omitted but of course not in the calculation of mol fraction of H_2O^{16}.

Considering $\frac{dc}{dx} =$ constant throughout the stomach wall the diffusion law can be written $\frac{dn}{dt} = -\frac{DA}{\triangle x} \cdot \triangle c$, where $\triangle x$ is the thickness of the wall and $\triangle c$ the concentration difference between the two sides. Putting $\frac{DA}{\triangle x} = k'$, a permeability coefficient (cm^3 sec^{-1}) is obtained, which was calculated according to

$$k' = \frac{V_i V_o}{(V_i + V_o)t} \cdot 2.3 \log \frac{V_i C_i + V_o C_o}{V_o (C_o - C_i)}$$

where V_i and V_o are the volumes inside and outside the stomach, C_i and C_o the concentration in these volumes at time t.

If k' be divided by the surface area A of the stomach a more important expression of the permeability coefficient k (cm sec^{-1}) is obtained. This was done under the assumption that the stomachs resembled a part of a rotation ellipsoid with the long axis (rotation axis) twice the short one. Then the surface area A could be estimated from the volume V (put equal to the weight of stomach and its content) from

$$A = 6.08 \cdot V^{2/3}.$$

As the water had to penetrate the entire stomach wall the estimation of the surface area was not invalidated by the complexity of the structure of the mucosa.

From 43 experiments the k-value for HDO became $(1.49 \pm 0.08) \cdot 10^{-5}$ cm sec^{-1} and from 25 expts that for H_2O^{18} $(1.91 \pm 0.09) \cdot 10^{-5}$ cm sec^{-1}.

Before proceeding we have to account for the fact that H_2O^{18} shows a higher permeability coefficient than HDO. According to WANG, ROBINSON and EDELMAN (1953) the self diffusion coefficient of H_2O^{18} in H_2O^{16} is about 14 % higher than that of D_2O in H_2O^{16}. In 17 experiments where HDO and H_2O^{18} were used simultaneously the quotient k_{O18}/k_D was calculated for each experiment and gave an average value of 1.29 ± 0.07 *i. e.* not very significantly different from Wang's figure. (Note that $k_{O18}/k_D = D_{O18}/D_D$ when the two isotopic waters are used simultaneously on the same stomach.) Now it should be remembered that the experiments were performed with different concentrations in HDO and H_2O^{18}. Usually about 5 mol % D (*i. e.* 10 mol % HDO) was used but only 0.075 mol % O^{18} in excess. In the latter case we may consider the labelled water molecules to diffuse individually and the obtained diffusion coefficient to be equal to the real "tracer diffusion coefficient" (cf. WANG 1952). In the case of HDO, however, a much higher concentration was diffusing into H_2O^{16} and the diffusion coefficient was probably not constant along the diffusion pathway. Thus an integral diffusion coefficient was obtained which would yield too low values compared with the "tracer diffusion coefficient", with the consequence that D_{O18}/D_D will be too high. From Wang's papers (WANG 1951 a, b and WANG, ROBINSON and EDELMAN 1953) a quotient of 1.34 is obtained if an integral diffusion coefficient is used for the deuterium water.

As in my experiments the diffusion was studied in salt solutions, which will alter the diffusion coefficients (WANG 1954) and at 37° C, which may change the quotient due to different activation energies for the two types of water, it was thought necessary to perform similar experiments with an artificial neutral membrane. For this purpose a Stokes' sintered glass diaphragm cell (STOKES 1950) with two compartments containing about 10 ml each was used. Both compartments were filled with Krebs solution but to the lower one also isotopic water was added. The cell was mounted in a 37° C waterbath. Two hours were allowed for the diffusion

gradient to be established across the membrane and then the upper compartment was renewed and the experiment started. By this method the ratio of the two diffusion coefficients $D_{O^{18}}/D_D$ became 1.19.

As the results from the stomachs do not significantly differ from those in the diffusion cell the faster penetration of H_2O^{18} than of HDO through the gastric mucosa must be a result of an inherent property of the water molecules and not a result of stomach activity.

When HDO was added to the outside and H_2O^{18} to the inside of the stomachs the ratio $k_{O^{18}}/k_D$ did not differ from the other experiments, where the two isotopes penetrated in the same direction. This is what could be expected if no net transport of water takes place. Thus this is a check on the results cited above that no gain or loss in weight occurred.

As the two isotopic waters show different rates of penetration it is not permissible to use any of the obtained diffusion rates for calculation of the fluxes of ordinary water unless we know the relation between $D_{H_2O^{16}}$ and the diffusion coefficient of one of the other waters. It was therefore thought of interest to estimate the self diffusion coefficient for H_2O^{16} in water. Thus two Krebs solutions were prepared with pure D_2O (99.97 mol % D) and to 12 ml of one solution 0.1 ml of H_2O^{18} (containing 9.37 mol % H_2O^{18} and 100 mol % H) was added and to 12 ml of the other the same volume of D_2O. The two solutions were placed in the Stokes' cell. Only one experiment could be performed and no time was allowed for gradient stabilization. By simultaneous determination of H_2O^{16} and H_2O^{18} penetration it was possible to calculate $D_{O^{18}}/D_H$ which became 0.52. This figure is, however, very uncertain because the equilibrium constant for the reaction $D_2O^{18}(l) + CO_2^{16}(g) = D_2O^{16}(l) + CO^{16}O^{18}(g)$ is not known but the same constant was used as for $H_2O^{18}(l) + CO_2^{16}(g) = H_2O^{16}(l) + CO^{16}O^{18}(g)$. One can only state that the self diffusion of ordinary water is at least as great as that of H_2O^{18}.

Discussion.

In agreement with earlier observations (Cope, Blatt and Ball 1943, Crane, Davies and Longmuir 1948 and Code, Scholer, Hightower, Dietzler and Baldes 1954) it was found that water penetrates the stomach mucosa very readily. Simultaneous use of HDO and H_2O^{18} in mouse stomachs revealed permeability coefficients, k, of $1.49 \cdot 10^{-5}$ cm sec^{-1} and $1.91 \cdot 10^{-5}$ cm sec^{-1} re-

spectively. This is of the same order as for frog skin ($7.3 \cdot 10^{-5}$ cm sec^{-1}) as shown by GARBY and LINDERHOLM (1953).

As $k = \frac{D}{\triangle x}$, where D is the diffusion coefficient, and $\triangle x$ the thickness of the membrane, the permeability coefficient could be calculated if D and $\triangle x$ were known. From WANG, ROBINSON and EDELMAN (1953) D_{37° for H_2O^{18} was calculated to $3.65 \cdot 10^{-5}$ cm^2 sec^{-1}. If the thickness of the stomach is roughly estimated to be 0.05 cm,

$$k = \frac{3.65 \cdot 10^{-5}}{0.05} = 73 \cdot 10^{-5}$$

This figure would appear if the diffusion had taken place in a water filled pore of 1 cm^2 area. Compared with the obtained k-value $1.91 \cdot 10^{-5}$, it shows that $\frac{1.91}{73} = 0.026$ or about 3 % of the total mucosal area would be the water filled pore-area, provided that the water mainly penetrated by intercellular routes. This fractional pore-area is less than for the glomerular membrane, which was calculated to be 0.08 by PAPPENHEIMER (1955) or 0.17—0.33 by GARBY and WALLENIUS (1955) but much higher than that of muscle capillaries, which was found to be less than 0.002 by PAPPENHEIMER, RENKIN and BORRERO (1951). Judging from the high figure obtained it is therefore quite probable that the water penetrated not only intercellularly but also through the cells.

Total flux of water.

The dimension of the permeability coefficient, k is $cm^3 \cdot sec^{-1}$ per cm^2 membrane area and in the case of water it expresses a real volume of water that has penetrated. To obtain the net flux according to Fick's law we use $\frac{dn}{dt} = k\,(C_o - C_i)\,\frac{M_w}{1{,}000\ d}$ where C_o and C_i are the molarities of water in the outside and inside solution, M_w is the molecular weight of water and d its specific gravity. This gives us the flux $\frac{dn}{dt}$ in ml $cm^{-2} sec^{-1}$.

The mean value of $k_{O^{18}}$ was $1.91 \cdot 10^{-5}$ cm sec^{-1}, *i. e.* 0.019 μl/sec or 1.10 μl/min or 0.069 ml/hr could penetrate 1 cm^2 of the stomach wall in either direction. Of course an error (but in these calculations unimportant) may be introduced when a "tracer

diffusion coefficient" is used for a calculation of the partial fluxes of "concentrated" water. The only figure on the water permeability in the stomach seen in the literature was mentioned by HOGBEN (1955) as unpublished results (1.5 ml $cm^{-2}hr^{-1}$). His figure is about 20 times greater, but was probably obtained from the frog mucosa, which in the present experiments appeared to admit a more rapid penetration of water than that of the mouse. In this connection it ought to be remembered that a great part of the mouse stomach consists of a non-glandular wall.

If the water penetrates the cells without interaction between the molecules the total unidirectional flux may also be equal to the maximal net flux if the water concentration on one side approaches zero *i. e.* Fick's law may be used to calculate the flow of water due to osmotic pressure differences. If water moves as a bulk flow, however, the net flux will be greater than effected by diffusion only, but from the foregoing it seems probable that water to a great extent penetrates *through* the cells. This is suggested also by the findings that the primary secretion contains no Na^+, but K^+ in a concentration a little higher than the extracellular fluid (LINDE and ÖBRINK 1950). This fact is hard to interpret as a result of a pure sterical hindrance for Na^+ and not for K^+ in intercellular pores. Thus one could possibly expect the maximal net flux of water to be 0.069 ml $cm^{-2}hr^{-1}$.

The average secretion of acid in the stimulated stomachs of mice was 20.5 μmol hr^{-1}. If a concentration of 170 mN is assumed for the primary acidity this corresponds to a volume secretion of 0.12 ml hr^{-1}. As the surface area as an average was 12 cm^2 this corresponds to 0.01 ml $cm^{-2}hr^{-1}$.

Compared with the maximal net flux of water calculated above — 0.069 ml $cm^{-2}hr^{-1}$ — it is obvious that the total secretion of water for the hydrochloric acid can be accounted for by sufficient osmotic forces.

In this connection it may be of interest to examine the results of REHM, SCHLESINGER and DENNIS (1953). They instilled hypertonic solutions in dogs stomachs and could not get an osmotic net transport of water sufficient to explain the normal secretion rate. Actually they got only about one tenth of the volume increment that they normally obtained during secretion. Therefore they concluded that the surface epitelial cells (SEC) are impermeable to water, which conclusion is one of the main features in their new hypothesis on gastric acid formation. In their experiments,

however, they used 0.32 M salt- or acid solutions as the most concentrated agents. That means that in the case of 0.32 M NaCl the water concentration was 98.85 mol % or 54.20 mols/l compared with 99.42 mol % or 54.76 mols/l in the plasma. Thus by using these figures for the stomach of the mouse and with the concept given above that the water penetrates the cells individually one would expect a net flux of water $= 1.91 \cdot 10^{-5}$ (54.76—54.20) 18.016/1,000 = 0.0002 μl $cm^{-2}sec^{-1}$ or 0.012 μl $cm^{-2}min^{-1}$ or 0.72 μl $cm^{-2}hr^{-1}$, which is a rate about 13 times lower than the obtained secretory rate (0.01 ml $cm^{-2}hr^{-1}$). Furthermore the solutions which they instilled could also diffuse through the gastric mucosa thus causing an even lower net transport of water. Therefore by the method of Rehm one could not expect a higher net flux of water even if the SEC are so readily permeable to water as to allow sufficient water to penetrate to explain the normal volume secretion.

Influence of water flux on the ionic concentrations:

As has been conclusively demonstrated, the chloride concentration varies with the secretion rate so as so give a hypotonic juice at a fairly low secretion rate (Gilman and Cowgill 1931, 1933, Lifson, Varco and Visscher 1941, 1943, Teorell 1947 and Öbrink 1948). Maximally this hypotonicity is about 30 milliosmols, and the secretion rate about 2—5 % of the maximal rate, *i. e.* 0.012—0.03 ml $cm^{-2}hr^{-1}$ in a dog or cat. This hypotonic juice would of course tend to give a net transport of water that in the mouse stomach would be equal to $-1.91 \cdot 10^{-5}$(54.76—54.78). $\frac{18.016}{1{,}000} = 6.9 \cdot 10^{-8}$ ml cm^{-2} sec^{-1} or $2.5 \cdot 10^{-5}$ ml $cm^{-2}hr^{-1}$ which probably is of little importance compared to the secretion rate. Consequently this tendency to oppose the fluctuations in tonicity of the gastric juice is probably unimportant in the evaluation of the real permeability coefficients for the different ions.

Comparison of $k_{O^{18}}$ and k_{HCl}.

In mice the permeability coefficient for instilled HCl measured as H^+ was 0.0115 cm $hr^{-1} = 3.19 \cdot 10^{-6}$ cm sec^{-1}, *i. e.*, $k_{O^{18}}/k_{HCl} = 6$.

From the results of heavy water absorption experiments by Cope, Blatt and Ball (1943) and Scholer and Code (1954),

permeability coefficients were roughly estimated and compared with the permeability coefficient for HCl through the gastric mucosa by ÖBRINK (1948) and TERNER (1948, 1949 a, 1949 b). This comparison gave a quotient of the same order as in the stomachs of the mice.

Water flux in stimulated and nonstimulated stomachs.

No difference in water permeability between stimulated and nonstimulated stomachs could be shown but such a difference may still exist in individual types of cells though it vanishes in the overall water permeability. Other types of experiments have to be tried to examine this particular problem.

Summary.

In isolated mouse stomachs filled with Krebs solution to a pressure of 75 cm H_2O and incubated in 37° C and 4.2 atm. oxygen pressure (technique of Davenport) no net transport of water takes place in spite of a fairly high acid secretion. In such preparations it was found by the simultaneous use of HDO and H_2O^{18} that the stomach wall is readily permeable to water. The permeability coefficients were $1.49 \cdot 10^{-5}$ cm sec^{-1} for HDO and $1.91 \cdot 10^{-5}$ cm sec^{-1} for H_2O^{18}. The difference in the two figures is dependent on inherent physical differences in the two water molecules. It was thought that the normal volume secretion could be explained from these permeability figures and that the transportation of H^+ or Cl^- would exert sufficient osmotic forces for the normal water transport during secretion.

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From the Institute of Occupational Health, Helsinki.

Lack of Effect of Oxygen Tension upon the Permeability of the Guinea Pig Placenta to d(-|-)xylose and Sorbose.

By

M. J. KARVONEN, A. LAAMANEN and N. RÄIHÄ.

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The rate at which different sugars cross the placenta varies from sugar to sugar. The aldoses glucose and d(+)xylose are examples of rapidly crossing sugars, whereas the ketohexoses sorbose and fructose pass the placenta slowly. It has been assumed that the permeability of the placenta to d(+)xylose is of a similar nature as that to glucose; the permeability to sorbose is comparable with that to fructose (KARVONEN et al., in press). D(+)xylose and sorbose may thus be used as tracer analogues to glucose and fructose, respectively, due to the inability of the organism to metabolize the foreign sugars.

The purpose of the present study was to compare the placental permeability of sugars under normal conditions to those existing when the mother is kept under hypoxia or hyperoxia, by making it breathe different oxygen-nitrogen mixtures.

Material and Methods.

Guinea pigs were used as experimental animals. They were anaesthetized with Nembutal intraperitoneally. The foetuses were delivered through caesarean section into a 0.9 % NaCl bath kept at 38° C, where they remained connected with a functioning placenta. The foetuses were weighed after the experiment; their exact age was not known.

In all experiments 1 ml of a 10 % sugar solution was injected into the foetal circulation. The sugar was injected into that part of the umbilical vein which runs intra-abdominally in the foetus. All the blood samples were also taken from this vein. This technique decreased the tendency to obstruction of the umbilical vein, as has been pointed out by HUGGETT for sheep (personal communication). The maternal blood samples were collected from the carotid artery.

Oxygen-nitrogen mixtures were supplied to the mother through a tracheal cannula which was connected to a gas cylinder containing 10 % oxygen + 90 % nitrogen in the hypoxia experiments, and 80 % oxygen + 20 % nitrogen in the hyperoxia experiments. A pressure equilibrator consisting of an inner balloon of a football was placed between the gas cylinder and the tracheal cannula. A small opening in the tracheal cannula allowed the expired air to escape. The gas was administered at a pressure of c. + 5 mm H_2O. The whole system was set up in such a way that the gas mixtures could be changed in the middle of the experiment. Switching from one gas mixture to the other caused a marked change in the colour of the maternal and foetal blood. When the change from low to high oxygen concentration was made, the colour of the placenta changed much more slowly than that of other organs.

As the guinea pig usually gives birth to more than one offspring, the experiments were performed on half of the foetuses of each mother during hypoxia, leaving the rest untouched, and changing over to hyperoxia before the remaining foetus or foetuses were exposed. The order of the hypoxia and hyperoxia experiments was varied.

The blood samples were deproteinized with cadmium hydroxide precipitation (FUJITA and IWATAKE 1931). Sorbose was determined by using an indol method (KARVONEN and MALM 1955). For determining xylose, glucose was removed by absorption to baker's yeast: five volumes of filtrate was incubated with 1 volume of a 1 : 10 suspension of washed yeast, at room temperature for 4 hrs. Xylose was then determined from the yeast-treated filtrate by using SOMOGYI's (1952) method.

Results.

D(+)xylose. The table gives the results of the experiments, in which one of the twin foetuses was given a xylose injection during hypoxia and the other during hyperoxia. Three such experiments were performed. The table also presents quantitative data from corresponding experiments made while the mother was breathing ordinary air.

The rate of the disappearance of the injected sugar from the foetal circulation is expressed as "apparent net transfer rate" (ANTR) which indicates change of foetal blood sugar level per minute, per unit foetal-maternal gradient (KARVONEN and RÄIHÄ 1954).

Table.

The rate of disappearance of injected D(+)xylose or sorbose from the guinea pig foetus, while the mother was breathing air, 80 % O_2 or 10 % O_2 in nitrogen. ($\bar{p} = \frac{p_1 + p_2}{2}$; *other symbols are explained in the column headings.*)

No. of exp.	Foetal weight g	Gas mixture	Interval of samples t	Change of foetal sugar mg% $p_1 \rightarrow p_2$	Rate of change mg%/min. $\frac{p_1 - p_2}{t}$	Mean maternal level mg% P	Apparent net transfer rate min^{-1} $\frac{p_1 - p_2}{t} \times \frac{1}{\bar{p} - P}$
D(+)xylose							
1	83	Air	11′ 10″	179→ 75	9.32	16	8.4×10^{-2}
			11′ 30″	75→ 43	2.78	29	9.3×10^{-2}
2 I	70	80 % O_2	5′ 30″	210→105	19.1	28	14.7×10^{-2}
II	76	10 % O_2	4′	215→146	17.3	39	12.2×10^{-2}
3 I	84	80 % O_2	10′	235→124	11.1	27	7.3×10^{-2}
II	86	10 % O_2	10′	294→123	17.1	45	10.4×10^{-2}
4 I	102	80 % O_2	9′	241→133	12.0	32	7.7×10^{-2}
			7′	133→ 81	7.43	30	9.6×10^{-2}
II	98	10 % O_2	10′	172→ 68	10.5	26	11.2×10^{-2}
Sorbose							
5	94	Air	5′	203→191	2.4	4	1.2×10^{-2}
6	51	80 % O_2	10′	438→396	4.20	10	1.0×10^{-2}
7 I	96	80 % O_2	10′	280→243	3.70	5	1.4×10^{-2}
II	86	10 % O_2	28′	287→223	2.28	20	1.0×10^{-2}
8 I	125	80 % O_2	10′	213→170	4.30	7	2.3×10^{-2}
			12′ 30″	170→121	3.92	9	2.9×10^{-2}
II	125	10 % O_2	10′	235→184	5.10	6	2.5×10^{-2}
			14′ 30″	184→138	3.17	11	2.1×10^{-2}

The results show that d(+)xylose disappears relatively fast from the foetal circulation, and that the rate is essentially unaffected by variations in the oxygen tension within the range used.

Sorbose. Sorbose disappeared from the foetal circulation more slowly than xylose. The results of each experiment are listed in the Table. Again, no systematic change of the rate of transfer was effected by varying the oxygen tension of the gas which the mother had to breathe.

Discussion.

The present results show that changes in the oxygen tension on the maternal side of placenta within the range used have no radical effect upon the placental transfer of either d(+)xylose or sorbose; of these xylose is an example of rapidly crossing and sorbose of slowly crossing sugars.

Considerable variation was observed in the rate of transfer from one experiment to the other. It has been shown that the transfer rate is the slower, the higher the concentration of the sugar on the side of the elevated concentration (WIDDAS 1952; KARVONEN et al., in press); however, the effect of this variation was minimized in the present series by always using the same dose of sugar.

Another of the factors probably causing variation between the individual experiments was the blood glucose level. As it has been shown that fructose and glucose compete of the same carrier system (HUGGETT 1954), uncontrolled variations in the glucose level may be expected to affect the transfer of other sugars. However, it is difficult to judge whether this circumstance caused any scatter in the present experimental setup.

It has been shown that the permeability of placenta to a series of sugars is essentially analogous to the permeability of human erythrocytes to different pentoses and hexoses (KARVONEN et al., in press). If one wants to study the effects of varying oxygen tension on this type of carrier system in general, the use of erythrocytes instead of placenta *in situ* would offer two advantages: a better control of the experiment, and the feasibility of testing a wider range of oxygen tensions, down to zero.

Biologically the circumstance that the placental transfer of sugar is not affected in either sense by low oxygen tensions may contribute to the survival of the foetus under adverse conditions. Glucose is able rapidly to cross even an anoxic placenta. The foetus is adapted to fast rates of anaerobic metabolism, and as the anaerobic breakdown of sugar releases only a small portion of the total energy of carbohydrate, a fast supply of sugar is needed. The foetus is also able to use its placenta for the elimination of the acid metabolites resulting from the anaerobic processes (KARVONEN et al., 1949), and thus is able to survive to quite an important extent without oxygen, so long as it has a functioning placenta which supplies glucose and eliminates lactate and other metabolites.

For the foetuses of ungulates, which have considerable amounts of fructose in their blood, a relative impermeability to fructose is probably equally important in hypoxia. The glucose level of *e. g.* a sheep is generally of the order of c. 40 mg per cent, but may fall to extremely low values, under 10 mg per cent, under adverse conditions. For its anaerobic metabolism, the foetus cannot count

on sufficient supply of maternal glucose. Thus it is important for it that its fructose reserve does not leak into the mother even during placental hypoxia.

Summary.

The effect of changes in oxygen tension upon the permeability of the placenta to d(+)xylose and sorbose was studied in situ. D(+)xylose or sorbose was injected into the foetal circulation and the fall in the foetal blood sugar was studied.

It was observed that a maternal hypoxia or hyperoxia had no clearcut effect upon the placental transfer of these sugars.

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Biological Institute, Carlsberg Foundation, Copenhagen, Denmark.

The Plasminogen Activator in Animal Tissue.

By

TAGE ASTRUP and IDA STERNDORFF.

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Plasmin, the proteolytic enzyme of blood, is formed by activation of its precursor plasminogen. This activation can be effected by a variety of activating agents, for references see ASTRUP (1955, 1956). Schematically the interaction can be presented as follows:

plasminogen ⟶ plasmin

↑

activators of plasminogen: spontaneous, chloroform, trypsin, tissue activator ("fibrinokinase"), urine activator, activator in blood, milk etc.

The plasminogen activator in animal tissue was originally called *fibrinokinase*, ASTRUP and PERMIN (1947), in analogy with the term streptokinase used for the activating agent produced by streptococci. When it had been found that streptokinase does not activate plasminogen directly, but acts through an intermediary stage (MÜLLERTZ and LASSEN 1953), the term fibrinokinase was given up and replaced by the neutral term *tissue activator of plasminogen*. This name does not involve any commitments as to the mode of action.

The tissue activator is a fairly stable compound contained in the structural proteins of the cytoplasm (PERMIN 1947). It cannot be brought into solution by ordinary means, but it can be extracted by thiocyanate solutions (ASTRUP and STAGE 1952). In contrast to the tissue activator, the activating agents found in urine or formed in blood and other fluids in the organism are very labile compounds, easily soluble in aqueous solutions (ASTRUP and STERNDORFF 1952 (urine), MÜLLERTZ 1955 a and b (blood), STORM 1955 (lachrymal fluid)). Evidently two different kinds of plasminogen activators exist in the organism. It is the purpose of this paper to report some experiments with the tissue activator substantiating its difference from the above mentioned activating agents of the labile type.

Experimental.

1. Estimation Method.

Activator activity was estimated on standard fibrin plates with 0.1 per cent fibrin (bovine, containing plasminogen, ASTRUP and MÜLLERTZ 1952), and recorded as diameter products of the lysed zones. The presence of 1-M KSCN in the test solutions did not interfere significantly with the activity estimations.

2. Extraction of the Tissue Activator.

Muscular tissue from pig hearts was treated with water and saline and the residue dehydrated with acetone as previously described (ASTRUP 1951 b). Extraction of the air dried powder was performed with M potassium thiocyanate by shaking for 2 hours (10 ml per g). After pressing through a double layer of gauze, the brownish and turbid extract was filtered. No increase in activity of the extract was obtained by increasing the extraction time, but repeated extractions gave active solutions of decreasing activity. After five extractions, no activity was left in the tissue residue, as indicated by the absence of fibrinolytic activity when samples of the residue were placed on standard fibrin plates. Usually only the first two solutions were pooled and used for further experiments although the extraction was far from quantitative.

A precipitate containing part of the activity separated during dialysis of the crude extract against tap water, saline or buffers. An active dry powder could be precipitated with acetone (ASTRUP and STAGE 1952). When KSCN was added to the dialyzed suspension to the concentration of 1-molar, the total activity was found in the supernatant.

3. Acid Precipitation.

All activity could be precipitated from the KSCN-solution by addition of 0.1 vol. 0.1-n sodium sulphosalicylic acid followed by 1-n HCl until pH 4.0. The precipitate did not dissolve completely in 0.9 % NaCl

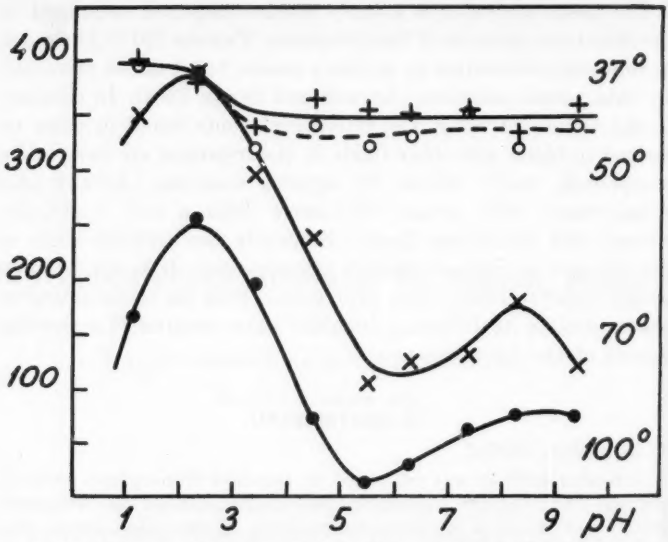

Fig. 1. *Thermostability of a soluble plasminogen activator from pig heart.* *Abscissa*: pH of solutions during heating for 30 mins. at various temperatures. *Ordinate*: Activity of heated and neutralized solutions.

or in buffers, but all activity went into solution in M-KSCN after neutralization with a few drops of 1-n NaOH. After cooling, 3 vol. of ice cold acetone were added, and the precipitate dried with acetone and ether. A yield of 3—6 mg of powder was obtained per ml original solution, containing 40—60 per cent of the activity. The product did not dissolve completely in 0.9 % NaCl or buffers (phosphate, pH 7.6 or borate, pH 7.8). After centrifugation, the active supernatant could again be precipitated with acetone with slight loss of activity, yielding a yellowish, water soluble powder.

4. Thermostability of the Heart Tissue Activator.

Solutions of the soluble activator in appropriate buffers were heated for 30 mins. After neutralization with solid $NaHCO_3$ or KH_2PO_4, the activity was estimated. Some solutions remained turbid after neutralization. Fig. 1 shows that the tissue activator is fairly stable, especially at acid reaction. This experiment was made with a solution prepared by dissolving the acid precipitate directly in phosphate buffer (pH 7.6, 0.1 M) with no acetone precipitation. Acetone powders gave similar curves.

5. Pig Lung as Plasminogen Activator.

Washed and acetone treated pig lung tissue produces activation of plasminogen (ASTRUP 1952), and was therefore investigated as a source of the soluble tissue activator. Solutions nearly as active as the heart

tissue extracts could be produced by treatment with 1-M KSCN. However, the lung tissue swelled considerably more than the heart tissue during this treatment. A dry powder could be prepared, as described for the heart tissue activator; however, the losses in activity encountered during the procedures were higher for the lung tissue.

Discussion.

When comparing the properties of the tissue activator of plasminogen, as here described, with the properties of the urinary activator (ASTRUP and STERNDORFF 1952), or the activators formed by addition of streptokinase to human blood (MÜLLERTZ 1955 b), to human lachrymal fluid (STORM 1955), saliva (ALBRECHTSEN and THAYSEN 1955) or amniotic fluid (ALBRECHTSEN and TROLLE 1955), it is evident that the tissue activator is of a different type. It can be treated chemically in ways which destroy the above mentioned activators. The thermostability curves especially show a clear difference. The tissue activator is a fairly stable substance, resistant to heating for 30 mins. at 37° or 50° over a broad pH range. In acid solutions it is fairly stable even at 70°. This is contrary to the behavior of the activators mentioned, which are especially labile at acid reaction, being destroyed at 37°. The streptokinase activated blood activator (MÜLLERTZ 1955 b) is apparently more labile than the streptokinase activated activator in lachrymal fluid (STORM 1955), but different preparations and estimation methods were used in these two instances. The urine activator is apparently slightly more stable than the blood activator, especially at alkaline reaction (ASTRUP and STERNDORFF 1952). These minor differences might be caused by differences in the contents of contaminating proteins, including inhibitory agents, which also are influenced by heating, and which interfere to a varying degree with the different estimation methods. However, the tissue activator differs so much in stability from these activators, even regarding the minor differences mentioned, that it clearly represents a different type of chemical compound. The slight increase in activity observed in Fig. 1 at very acid reaction might be caused by destruction of contaminating inhibitory agents.

It appears appropiate to recall that the tissue activator possibly activates plasminogen directly by a stoicheiometric reaction (ASTRUP 1951 a), while the activator formed from a precursor in blood probably activates plasminogen by an enzymatic reaction

(MÜLLERTZ 1955 b). These observations together with the above mentioned chemical properties emphasize the differences between the two types of activators and the ability of the organism to produce proteolytic activity by means of two different processes, namely a process with a localized effect (the tissue activator) and a process of more general character (the blood activator). Perhaps it is of physiological significance that the tissue activator is fairly stable, while the catalytically acting blood activator is easily destroyed. This property might be useful for the organism when a rapid destruction of a potent compound is needed in order to keep the interactions under control.

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Summary.

1. The plasminogen activator in pig heart tissue and pig lung tissue has been isolated in soluble form and partially purified.

2. The tissue activator is a fairly thermostable substance especially at acid reaction.

3. The tissue activator represents a type of plasminogen activator different from the activator found in urine or produced in blood, milk, and a number of other fluids in the organism.

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From the Laboratory of Clinical Physiology, Serafimerlasarettet, Stockholm.

The Effect of Posture upon the Equilibrium between Carbon Monoxide in Rebreathed Gas and the Carboxy Hemoglobin Concentration of the Blood.

By

HANS DAHLSTRÖM.

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Inspired gas is unevenly distributed over the alveolar spaces of the lungs (FOWLER 1952, ROOS, DAHLSTRÖM, and MURPHY 1955). The pulmonary artery blood is also unevenly distributed to the alveoli (WEARN, et al. 1934). The effects of these factors upon the alveolar-arterial O_2 difference were discussed by FAHRI and RAHN (1955), who postulated a normal distribution of the quotients ventilation/perfusion of the alveoli. They pointed out that when 100 % O_2 is breathed, the venous blood in an unventilated alveolus will become oxygenated, and the O_2 uptake will continue. The P_{O_2} of the end-capillary blood will be approximately the same for all degrees of ventilation of the alveoli. As soon as small fractions of N_2 are present in the inspired mixture, the composition of the gas in the non-ventilated perfused alveolus will rapidly move toward equilibrium with the venous blood. Consequently, the blood from a non-ventilated alveolus will contribute venous blood to the arterialized blood in the same manner as a direct shunt.

It would appear probable that if shunts of this nature exist to an appreciable degree, they would affect the equilibrium between CO in a rebreathing system and the COHb concentration of the blood. The lack of agreement between the COHb concentration

obtained by analyses of systemic blood and the values calculated from the conventionally estimated composition of alveolar gas (DAHLSTRÖM 1955) could possibly be attributed to such shunts.

Induced changes in pulmonary blood volume might influence the distribution of the ventilation/perfusion ratios, and such changes might therefore influence the equilibrium between the CO in a rebreathing system and the COHb of the blood. Changes of position, from the standing to the supine position, for example, are known to influence the pulmonary blood content (KJELLBERG, RUDHE, and SJÖSTRAND 1951). COMROE et al. (1955) stated that there is good evidence that a change in posture alone will cause some uneven distribution of blood throughout the lungs because of gravitational forces. From these aforementioned considerations it would seem that investigations concerning the influence of positional changes on the CO—COHb equilibrium would be of value.

Methods.

A group of healthy volunteers were selected for the experiments. A rebreathing apparatus for determination of total hemoglobin was used for most of the experiments (SJÖSTRAND 1948, NORLANDER 1955). The subject respired through a mouthpiece. The apparatus and the lungs of the subject were washed out with O_2 by 5 minutes of open system breathing, whereupon the rebreathing started. The expansible part of the system consisted of a rubber bag attached to a manifold, so that one bag could be removed and replaced by another without interruption. After the subject had rebreathed for $7^1/_2$ minutes the rubber bag was refilled with O_2. At the end of an additional $7^1/_2$ minutes the subject was requested to expire deeply, and the bag was removed for analyses. The rebreathing then continued in the same manner in a new bag previously filled with O_2, for another 15 minute period, and in several experiments, for an additional third period.

In other experiments a spirometry apparatus was used. A plastic bag allowed for expansion and a blower rather than valves directed the air flow. The respiration was recorded throughout the procedure and O_2 was supplied continuously to match the O_2 consumption. At the end of a 15 minute period a vital capacity manœuvre was performed, whereby about 5 liters of gas were expired into an empty bag, directed by means of a three-way stopcock. This bag was then removed and the contents analyzed.

During each 15 minute period the subject either stood, sat or lay in the supine position. In two experiments cuffs placed on the upper thighs were inflated above systolic pressure by means of storage tank pressure.

Analysis of the CO concentration was made in the CO-meter (SJÖSTRAND 1948), which was calibrated for each determination with a mixture of about 0.01 % CO in air which was taken from a large

storage tank which supplied the gas used throughout the experiments. The concentration of the calibrating gas was determined by diluting 96.8 % CO (manufacturer's specifications), according to a technique described earlier (DAHLSTRÖM 1955). The results were in general agreement with the values obtained with the iodine pentoxide method (Aga Ltd), and conformed to the calibrations from other institutions. The maximal error in the absolute value of the calibrating gas should be less than ± 6 %. It was estimated that when two consecutive samples with a CO concentration of about 2×10^{-5} are to be analyzed, a difference of 2 % or more would be significant.

Analysis of O_2 and CO_2 was performed by the Haldane method with an error of ± 0.2 % and ± 0.02 % respectively..

Results.

The five minute wash-out period with O_2 and the first 15 minute rebreathing period brought the N_2 concentration to between 5.2 and 2.6 %. The substitution of the sampling bag with a bag of O_2 at the beginning of the following rebreathing periods counteracted the increase in N_2 concentration resulting from an additional diffusion of N_2 from the blood and tissues. As can be seen from the tables, there is only a slight variation in the O_2 concentration during each experiment.

Table 1.

Results of gas analyses. Subject M. P. Blood volume apparatus. Three periods.

	Standing		Sitting		Supine	
	CO	O_2	CO	O_2	CO	O_2
	10^{-7}	%	10^{-7}	%	10^{-7}	%
A.	180	96.7	184	93.8	188	91.2
	216	97.4	238	96.2	232	97.5
	149	96.0	163	96.3	150	96.2
	178	96.5	182	97.4	184	94.8
	160	96.0	179	96.0	182	95.2
B.	209	97.2	246	96.1	236	95.4
	188	97.0	201	96.4	205	97.3
	136	95.0	143	94.7	150	95.7
	159	94.4	160	93.2	163	94.9
	148	95.6	158	96.2	161	94.8
Mean	172.3	96.2	185.4	95.6	185.1	95.3

A. Sequence: Standing, sitting, supine.
B. Sequence: Supine, sitting, standing.

Table 1 contains the results of experiments on subject M. P., a non-smoker. It can be seen that the CO concentration is higher in the sitting than in the standing position, regardless of the sequence of the experiments. The differences were tested by t-analysis, and found to be significant ($0.01 > P > 0.001$). The CO concentration in the supine position is also higher than in the standing position. This difference is also significant. In some experiments only slight changes among all three positions could be observed.

Table 2.

Results of gas analyses. Blood volume apparatus. Two periods.

Subject	Standing		Sitting	
	CO	O_2	CO	O_2
	10^{-7}	%	10^{-7}	%
TP	179	95.5	183	96.0
HE	250	96.4	257	91.7
MJ	172	95.7	191	97.2
DS	263	95.7	270	95.1
BW	163	95.8	180	95.1
MP	263	97.5	282	95.7
HD	590	95.5	639	97.8
	[1]605	96.0		
HD	473	96.0	486	94.1
HD	283	96.8	294	96.6
HD	462	97.9	468	95.2
HD	[2]323	89.7	355	93.6
Mean	311.0	95.7	327.7	95.3

[1] Values obtained during a third period, not included in the mean.
[2] Sequence: Sitting, standing.

Additional studies were performed on 6 subjects, some of whom were smokers (Table 2). The difference between the CO concentration in the standing and the sitting position is again significant. With one subject (H. D.), a third 15 minute standing period was additionally performed, again resulting in a decrease in the CO concentration. In another experiment on the same subject the sequence was reversed. In this instance the CO concentration was higher after the second rebreathing period.

In the experiments with the spirometry system (Table 3) samples of expired air were analyzed for CO, O_2, and CO_2. The usual increase of the CO concentration when the subject is seated can be

Table 3.

Results of analyses of expired gas. Subject HD. Spirometry apparatus employed. Two periods.

Standing			Sitting		
CO	O_2	CO_2	CO	O_2	CO_2
10^{-7}	%	%	10^{-7}	%	%
367	92.15	4.06	418	92.7	3.57
312	91.7	3.98	376	92.5	3.70

seen from these two experiments. The O_2 concentration of the expired air is increased by less than 1 % when the subject is seated. Although the alveolar O_2 concentration will be affected to a greater extent, the changes will still be less than 1 %.

Table 4.

Results of gas analyses. Subject HD. Blood volume apparatus. Three periods.

Standing		Sitting		Sitting	
CO	O_2	CO	O_2	CO	O_2
10^{-7}	%	10^{-7}	%	10^{-7}	%
446	96.7	[1]450	96.6	[2]470	95.3
392	97.4	406	95.9	[3]385	96.0

[1] Cuffs inflated before the subject was seated.
[2] Cuffs deflated at the beginning of the period.
[3] The subject having stood for 3 minutes, the cuffs were inflated and the subject seated.

Table 4 contains the results of experiments designed to demonstrate the influence of blood shifts to the lower extremities. After a 15 minute rebreathing period with the subject in the standing position, cuffs on the upper thighs were inflated immediately before the subject sat down. The CO concentration after the 15 minute rebreathing period in the seated position was practically unchanged. Thereafter, when the cuffs were released and another 15 minute period of rebreathing followed, a substantial increase in the CO concentration in comparison with the two previous periods could be recorded.

In another experiment the standing and sitting activities were performed as usual and followed by three minutes of standing,

whereupon the cuffs were inflated, the subject seated, and the 15 minute period completed. The usual increase of CO concentration occurred between the first two periods, while the last period was characterized by a return to the level of the first period.

Discussion.

The results indicate that in a rebreathing system employing a CO_2 filter, O_2 concentration of 95 % (approximately) and 14—45 $\times$ 10^{-6} CO, the CO concentration varies with the position of the subject. More CO is accumulated in the rebreathing system during the sitting or supine position than during the standing position. The changes are probably related to the redistribution of blood, for they can be abolished by occluding the circulation through the lower extremities.

It has been assumed that the equilibrium between CO in rebreathed gas and the COHb concentration in the blood follows Haldane's Law I, $(COHb)/(O_2\,Hb) = M \cdot P_{CO}/P_{O_2}$, wherein P_{CO} and P_{O_2} are estimated from the composition of inspired gas and corrected to alveolar composition by the assumptions that alveolar CO_2 = 40 mm of Hg and alveolar H_2O = 50 mm Hg. The experiments indicated that 5 to 6 % fluctuations of P_{CO} in the rebreathing system occurred during a change of the position of the subject from standing to sitting, or *vice versa*. The rebreathing system gained CO and the P_{CO} increased. According to the abovementioned assumptions, the COHb concentration of the subject should also have increased. This is an obvious contradiction.

Haldane's Law I pertains to conditions prevailing in the blood. The inconsistencies encountered in our results lead us to question the validity of the assumptions concerning P_{O_2} och P_{CO}. It is possible that the alterations of posture resulted in changes of both the pulmonary blood content and the distribution of the ventilation/perfusion ratios, of sufficient magnitude to alter the effective P_{O_2}. In this case, then, the distribution of CO between the blood and the rebreathing system would be affected.

The total volume of the rebreathing system and the lungs is about 13 liters. During the standing position the CO concentration decreases by 5 to 6 %. This implies that if the initial CO concentration is 250 $\times$ 10^{-7} (average for the determinations), approximately 0.018 ml of CO will be removed from the rebreathing system. The COHb of a subject having a blood volume of 5 liters and a CO

blood capacity of 20 volumes % will thereby be increased by 0.0018 %. Inasmuch as the initial COHb concentration is about 0.5 %, the increase of the COHb should be less than 0.5 % of the original value. Thus, in order to satisfy Haldane's Law I, it is necessary to postulate a change in the alveolar P_{O_2} or P_{CO} of about 5 to 6 %, which in the case of CO would be the reverse of the change observed in the rebreathing system. This is, of course, unexplainable. In the case of the P_{O_2} a regular change was not observed in the rebreathing system with positional changes nor was a decrease of this magnitude of the expired O_2 concentration obtained. The rather substantial decrease in the effective P_{O_2} which one is forced to accept must therefore be obscured by conventional methods of estimating the alveolar P_{O_2}.

At equilibrium we can postulate the following situation. The alveoli are schematically divided into two groups, well-ventilated and non-ventilated (Roos, Dahlström, and Murphy 1955). The P_{O_2} and the P_{CO} in the well-ventilated compartment is correctly estimated by the P_{O_2} in the rebreathing system and the conventional corrections for CO_2 and H_2O. The non-ventilated compartment, on the other hand has a lower P_{O_2} than the well-ventilated compartment, which results in a higher uptake of CO in the former. The systemic blood will therefore contain more CO than would have been the case had the conditions in the well-ventilated compartment prevailed. Accordingly, the mixed venous blood will carry a greater amount of CO to the well-ventilated alveoli than is contained in the end-capillary blood of this compartment. This will result in a continuous transfer of CO from the well-ventilated to the poorly-ventilated alveoli. The former, however, conduct a lively exchange with the rebreathing system. As a result the P_{CO} in the well-ventilated compartment of the lungs and the rebreathing system will be lower than that calculated in accordance with Haldane's Law I from the COHb concentration in systemic blood. There will be a continuous diffusion of CO from the well-ventilated to the non-ventilated compartment, the non-ventilated compartment supplying the blood with greater amounts of CO. This diffusion proceeds along a gradient between the P_{CO} in the rebreathing system and the end-capillary P_{CO} in the non-ventilated alveoli. The latter will not be directly proportional to the end-capillary COHb concentration, but will, according to Haldane's Law I, increase to a disproportionally greater extent with higher values of COHb. Consequently, the gradient will be relatively

higher at low values, facilitating the transfer of CO from the rebreathing system to the non-ventilated alveoli.

An explanation for the larger discrepancy between the P_{CO} in the rebreathing system and the COHb at low values can now be offered. In earlier calculations (CARLSTEN et al. 1954, DAHLSTRÖM 1955) high values of M were calculated from *in vivo* experiments at the low range of COHb (< 3 %). In the work of CARLSTEN et al., this observation particularly pertains to those values of COHb obtained either by the method of SIÖSTEEN and SJÖSTRAND (1951) or by a modification similar to the one employed by DAHLSTRÖM (1955). When the method of Horvath and Roughton was employed, highly aberrant results were obtained at the low range. With P_{CO} values less than 13×10^{-3} mm Hg, however, the mean value of M was 336, and the mean for the remaining values was about 250. In my own experiments (1955) I found a tendency for lower values of M to occur with higher levels of COHb. When a subject is in the standing position his lungs contain a smaller quantity of blood. It is conceivable that the diffusion paths may consequently be widened, facilitating the diffusion of CO from the well-ventilated to the poorly-ventilated compartments. Additional CO would then be taken up by the blood to the extent noted in these experiments. If the shift of blood is prevented by pressure cuffs on the thighs, the effect will not be observed.

It may be concluded, therefore, that when alveolar P_{O_2} is employed for calculations of the COHb concentration in accordance with Haldane's Law I, it may be overestimated by the conventional calculations. This ensuing error should be greater at low levels of COHb.

The evidence which appears to substantiate this possibility is as follows: (1), the values of the factor M, derived from *in vivo* determinations by Carlsten et al. (1954) and DAHLSTRÖM (1955), were higher than the traditional *in vitro* determinations; and (2), the values of M obtained at low levels of COHb are unexpectedly high, as observed in the data of CARLSTEN et al. (1954), and DAHLSTRÖM (1955). Curiously enough, the values of M obtained *in vivo* by LILIENTHAL et al. (1945) at very low arterial P_{O_2} are among the lowest reported.

It would therefore appear that values of M derived from *in vivo* determinations may vary with the pulmonary status.

The results of the effect of changes of position, although qualitatively uniform, vary quantitatively. In a few experiments the

differences in CO-concentration are well within the error of measurement, while in other experiments they amount to about 10 %. The calculation of COHb concentration from ventilation data, as employed by Sjöstrand's method for the determination of total hemoglobin, can therefore be expected to show a variance of up to 10 % with fluctuation of the pulmonary status.

It may also be concluded that both higher levels of COHb and lower O_2 concentrations could diminish errors in the calculation of COHb from rebreathed gas, since each of these factors will diminish the CO gradient between well-ventilated and non-ventilated alveoli.

Summary.

A closed circuit with a CO_2 filter, 95 % O_2 (approximately) and minute CO concentrations were used for rebreathing experiments in a group of healthy individuals. The CO concentrations appearing in the system were attributable to either endogenous formation of CO or tobacco smoking. The CO concentration in the system was found to vary with the position of the subject. Less CO accumulated in the system when the subject stood than when he sat or lay in the supine position.

A hypothesis was proposed concerning the nature of the equilibrium between CO in the alveolar spaces and the COHb concentration of the blood.

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From the Physiological Department, Faculty of Medicine
(Karolinska Institutet, Stockholm).

Neurotropic Effects of Substance P.

By

U. S. VON EULER and B. PERNOW.

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Substance P, a biologically active polypeptide found in extracts of intestine and brain (EULER and GADDUM 1931), has recently been obtained in a highly purified state (PERNOW 1953 a). Evidence has been produced showing that Substance P obtained from intestine and from brain are identical (ELIASSON, LIE and PERNOW 1956).

In a study on the distribution of Substance P in various parts of the brain of the dog PERNOW (1953 a, b) found large amounts in the hypothalamic region which has been confirmed by ZETLER and SCHLOSSER (1954) and AMIN, CRAWFORD and GADDUM (1954). Its presence in autonomic centres in the brain made it appear of interest to study whether administration to the centres by the vascular or intrathecal route would elicit actions in autonomically regulated functions. It was previously observed that injections of Substance P caused a stimulation of respiration (EULER and PERNOW 1954).

The present paper also includes a study of the effect of Substance P on autonomic reflex mechanisms and on autonomic ganglionic transmission as well as on the general behaviour of unanaesthetized test animals.

Methods.

Substance P was prepared from cow's intestine according to the method of PERNOW (1953 a). The preparations used contained 100—300 U. per mg and were dissolved in Ringer's solution immediately before use. The standard unit equals 7—10 threshold doses on guinea-pig's intestine in a 3 ml bath. For control purposes inactivated preparations were obtained by incubation with chymotrypsin.

The experiments were made on cats and rabbits. In the acute experiments the animals were anaesthetized with nembutal, 0.035 g intraperitoneally (cats), or urethane, 1.75 g/kg intravenously (rabbits). Respiration was measured by the body plethysmograph technique. Blood pressure was recorded from a femoral artery. In the diuresis experiments a poly-ethylene tube was inserted into the bladder and the urine secretion measured by an electronic drop counter.

The contractions of the nictitating membrane were recorded during supramaximal preganglionic stimulation for 15 sec. with a square wave stimulator, using a frequency of 28 and a pulse duration of 5 msec. Substance P solutions were injected intravenously by injection or infusion through the femoral vein, and intraarterially in the central direction through the lingual artery after tying the external carotid. Intrathecal injections were made intracisternally and into the third ventricle through a syringe needle fixed in a socket as described by DUNÉR (1953). Injections were also made into a side ventricle using the permanent cannula technique described by FELDBERG and SHERWOOD (1954). The location of the needle tip when injections were made intraventricularly was checked by the passage of cerebrospinal fluid through the needle and by injection of a dye, the distribution of which was studied post mortem. The volumes injected were usually 0.05—0.2 ml.

Cinematographic recordings were made on some occasions of the respiratory effects of intraventricularly injected Substance P in the unanaesthetized rabbit and of the general behaviour of the unanaesthetized cat with a permanent cannula in the side ventricle.

Results.

Rabbits.

a. *Intracisternal injections.* Intracisternal injections of 12 and 25 U. "P" in 0.05 ml saline in an unanaesthetized rabbit (K 1) caused a marked hyperpnoea which developed rapidly and remained for about $^1/_2$ hour on each occasion. In addition a strong vasodilatation was noted in the ears which became red and warm. After the larger dose definite signs of stupor were also noted, the animal lying with its head on the table. No convulsions or other signs were observed.

Intracisternal injection of 0.05 ml Ringer solution containing 25 U. "P" inactivated by chymotrypsin caused no visible effects.

b. *Intraventricular injections.* Similar results were obtained in two unanaesthetized rabbits when Substance P was injected into the III:rd ventricle through a needle which was inserted and fixed under local anaesthesia.

In rabbit K 2 the injection of 5—15 U. "P" in 0.05—0.15 ml Ringer solution caused after 5—10 min. hyperpnoea, which was

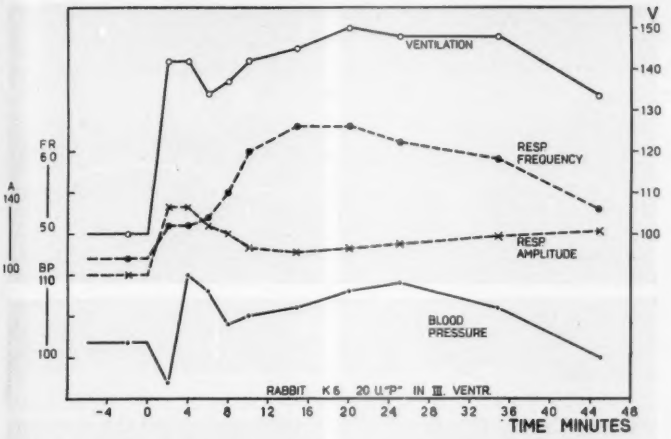

Fig. 1. Diagram showing the effects on blood pressure and respiration of 20 units of Substance P injected into the III:rd ventricle in the rabbit. Ordinates: A = respiratory amplitude, in % of original; FR = respiratory frequency, per min.; BP = blood pressure mm Hg.; V = ventilation % of original. Abscissa: time in min.

marked after the higher doses. Except for a discharge of 50 lumps of faeces no other symptoms were observed with certainty although some lack of spontaneity was noted. Control injection of Ringer solution (0.1 ml) in this as in other animals had no effect.

The experiment was repeated with larger doses in animal K 7. Injection of 30 U. "P" in 0.1 ml increased the respiratory frequency from 44 to 80 per min. No further effect on respiration was observed with 60 U. "P" which instead lowered the frequency from 80 to 50 per min. Pupillary dilatation was observed after this dose.

The effect of "P" on respiration and blood pressure following administration into the III:rd ventricle was recorded in three cases in anaesthetized animals.

A dose of 2 U. caused a small but definite increase in blood pressure and respiration. After doses of 5—20 U. "P" the respiratory frequency increased gradually by 25—75 % and then returned to normal in about 20—30 min. An increase in respiratory amplitude of 15—30 % was also observed. The blood pressure showed a sustained rise of 5—15 mm lasting for 10—30 min.,

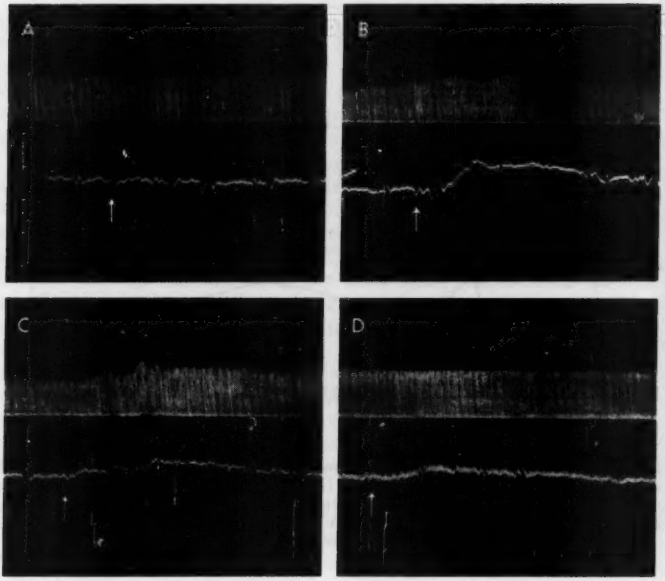

Fig. 2. Rabbit. Upper curves, respiratory movements recorded by body plethysmograph, lower curves blood pressure. Ordinate: 60—140 mm Hg. Injections of Substance P in III:rd ventricle. A. 5 U. inactivated, B. 5 U., C and D. 10 U. active "P". Time 30 sec.

preceded by a transient fall of some 40 mm Hg during 1—3 minutes in 2 of the animals.

The effects of a dose of 20 U. Substance P on blood pressure and respiration in rabbit K 6 is shown in Fig. 1.

An increase in respiratory frequency and amplitude of fairly long duration has thus been observed in the rabbit after intraventricular or intracisternal injection of Substance P in doses from 2 U. to 30 U. while 60 U. "P" reduced the frequency.

The blood pressure showed in all instances a small rise of relatively long duration, either starting immediately after the intrathecal injection or after a moderate fall.

On repetition of intraventricular injections of Substance P it was sometimes observed that the first dose caused a more marked action than subsequent ones. Fig. 2 illustrates the decreased response in animal K 5 to a dose twice as high as the first one.

Table I summarizes the results obtained on rabbits.

Table I.

Effects of Substance P on blood pressure and respiration in the rabbit.

+ = rise or stimulation; — = decrease or inhibition.

No.	Dose units	Administration	Blood pressure		Respiration	
			Transient (< 5 min.)	Longlasting (> 5 min.)	Transient (< 5 min.)	Longlasting (> 5 min.)
K 1	12	Intracisternally			+	+
»	25	»			+	+
K 2	5	III:rd ventricle			(+)	+
»	10	»			+	+
»	15	»			+	+
K 7	30	»			+	+
»	60	»			—	—
K 3	10	»			+	+
K 4	5	»	—	(+)	+	0
»	10	»	—	+	+	+
»	10	»	—	+	+	+
K 6	2	»	(—)	(+)	(+)	+
»	20	»	—	+	+	+
K 5	5	»	+	+	+	+
»	10	»	(+)	0	+	+
»	10	»	(+)	0	0	?

Cats.

a. *Blood pressure and respiration. Intraarterial injections towards centres.* Injection of 5 U. "P" intraarterially in the lingual artery in the central direction in animal C 1 caused a transient fall of blood pressure followed by a short-lasting rise. This was followed by a moderate, sustained fall in blood pressure from 150 mm to 134 mm after the first injection of 5 U. "P" and to 120 mm after the second injection of the same amount. The blood pressure then remained at this level for the rest of the experiment.

A short-lasting increase in respiration (frequency and amplitude) was also observed.

Intraarterial infusion of Substance P towards the centres at a rate of 2.6 U. per min. during 23 minutes (animal C 22) caused a marked stimulation of respiration but no immediate change in blood pressure. When 50 U. were infused 1 hour later at a rate of 6 U. per min. a transient inhibition of respiration and a fall in blood pressure of 40 mm Hg occurred at the beginning of the infusion. The blood pressure level then declined gradually, and after two subsequent infusions of 8 U. per min. for 8 min. and 15 U. per min. for 9 minutes the blood pressure had fallen from

145 mm Hg to 115 mm. Infusion of nicotine at a rate of 5 μg per minute for 6 minutes followed by 10 μg per minute for 10 minutes caused similar changes, inhibition of respiratory amplitude and a fall in blood pressure to 85 mm.

Intracisternal injections. Intracisternal injection of 7.5—12.5 U. "P" (C 2) caused a transient fall in blood pressure, followed by a short-lasting rise but not long-lasting effects. Respiration was moderately increased for a period of 5—10 minutes.

Intraventricular injections. Although the results of intraventricular injections were somewhat variable, in the majority of cases injections of 1—10 U. "P" in the III:rd ventricle caused a moderate, long-lasting stimulation of respiration preceded by a short period of inhibition. In 2 experiments inhibition only and in 2 experiments stimulation without inhibition were recorded.

The effects on the blood pressure were as a rule a transient moderate fall followed by a long-lasting small rise. In a few experiments no effects were found on the blood pressure, or only a decrease.

Repeated doses generally had a weaker, if any, effect, as previously described for intraventricular injections in the rabbit. Large doses usually caused only a transient increase of respiration and blood pressure.

The typical effects on intracisternal or intraventricular injections of doses of Substance P of 1—10 U. were thus a transient fall often followed by a prolonged small rise in blood pressure and an increase in respiratory frequency and amplitude sometimes preceded by a transient inhibition.

Table II summarizes the results obtained on cats.

b. *Effects on carotid occlusion reflex and on ganglionic transmission.* When a series of carotid occlusion tests were performed before and after injection of 25—50 U. of Substance P intravenously in the cat, no consistent change in the reflex rise of blood pressure was observed.

The contraction of the nictitating membrane as a result of preganglionic electrical stimulation was studied on cats before and after intravenous injections of Substance P. After doses of 100—200 U. "P" which caused a marked decrease in respiratory amplitude lasting for about 15 min., the contraction of the n. m. was unaltered or slightly depressed. A relaxation of the n. m. was noticed after 200 U. "P" (Fig. 3). No effects were noted on the control side.

Table II.

Effects of Substance P on blood pressure and respiration in the cat.

+ = rise or stimulation; — = decrease or inhibition.

No.	Dose units	Administration	Blood pressure		Respiration	
			Transient (< 5 min.)	Longlasting (> 5 min.)	Transient (< 5 min.)	Longlasting (> 5 min.)
C 20	1—10	Intravenous	—	0	+	(+)
»	100—200	»	—	0	(+)	—
C 1	5	Intraarterial (towards centre)	— +	—	+	0
C 22	2.5/min.	»	0	0	+ —	0
»	6-15/min.	»	—	—	—	0
C 2	7.5—12.5	Intracisternal	— +	0	+	(+)
C 6	1—5	III:rd ventricle	—	+	(—)	+
C 4	3	»	(—)	+	—	+
»	5	»	(—)	+	—	+
C 8	5	»	—	—	0	—
C 9	5	»	(—)	(+)	+	+
C 11	5	»	0	0	+	+
C 12	5	»	0	0	+	+
C 13	5	»	—	0	+	+
C 3	10	»	—	+	—	+
C 23	25	»	—	0	+	0
C 24	50	»	—	0	+	0
C 25	30	»	—	0	— +	0
»	50	»	—	0	+	+
C 15	30	Lateral ventricle			+	+

c. *Effects of injection of Substance P in the lateral ventricles of the cat.* Using the technique of a permanent cannula inserted in a lateral ventricle of the cat according to FELDBERG and SHERWOOD (1954) we have studied the effects of intraventricular injections in the unanaesthetized cat.

Control injections of 0.1—0.2 ml Ringer solution were never found to produce any visible effects in these respects. Substance P, however, in doses above 10 U. regularly caused a variety of symptoms, which were absent when similar quantities were injected after inactivation with chymotrypsin.

An increase in respiratory frequency and in respiratory depth was regularly observed with doses of 20—50 U. "P". Thus in cat C 15 a dose of 30 U. "P" in 0.15 ml solution caused an increase in respiratory frequency in 9 minutes from 36 to 64 per min.

d. *Behaviour.* In most cats receiving 20—50 U. "P" a lack of spontaneity was observed. Soon after the injection the animal lay down or stood still, frequently shutting its eyes. In contrast

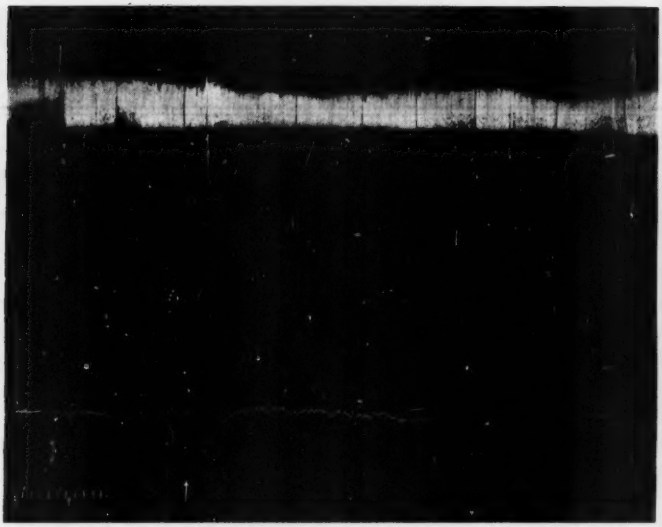

Fig. 3. Cat. Upper curve respiration, middle curve contraction of nictitating membrane on preganglionic stimulation for 15 secs., lower curve blood pressure (115 mm Hg at left). At arrow 200 U. Substance P injected intravenously. Time 30 sec.

to the general lack of activity the animal occasionally showed vigorous tail movements. At intervals it started moving about for a short while, apparently walking in a normal way. The usual spontaneity returned after about 1 hour. Licking movements were observed on several occasions.

In one of the cats (C 16) a marked change in the temper was noticed. After a dose of 20 U. slight aggressive tendencies were noted. A dose of 30 U. caused definite bad temper in this cat which was otherwise notoriously friendly. This attitude lasted for about $^1/_2$ hour. A still higher dose (50 U.) caused a state of moderate rage, the animal attacking any person coming near. This state lasted for more than 3 hours. During this time it was indifferent to food. The next morning the cat had returned to its "normal" friendly state.

In other cats which were similarly used for a series of intraventricular injections no reaction of this kind was noticed.

Acetylcholine in doses of 10 μg caused hyperpnoea, lack of spontaneity, vigorous tail movements, licking and yawning in

conformity with the observations of FELDBERG and SHERWOOD. These effects were not unlike those caused by Substance P. However, after the ACh injection the symptoms appeared almost immediately, while after "P" injection, the latency period was markedly longer.

When larger doses were used (100 U. "P") a state of stupor developed, lasting for about one hour and gradually receding. Except for vigorous licking movements no other symptoms were noticed.

e. *Effect on diuresis.* A series of experiments were performed in order to study any possible effects of Substance P on diuresis. Substance P was administered by various routes in the following doses:

Intravenous injection	50	—150	U.
» infusion	2	— 7.5	U./min.
Intraarterial infusion towards centres	2.5	— 7	U./min.
Injection in III:rd ventricle	5	— 50	U.

In no instance was any definite effect on diuresis noted. Injection of 100 U. intravenously was followed by a transient stop in urine secretion, lasting for about $1^1/_2$ minutes. The secretion then increased for a short period and then became normal again. After 150 U. the stop lasted for 4 minutes. During the next half hour bursts of urine at 2—4 min. intervals occurred, suggesting contraction effects on the ureters or the bladder. Control injections of nicotine caused a marked antidiuresis after a short latency period.

Discussion.

The experiments have shown that Substance P administered intrathecally produces certain effects on respiration and circulation. The most constant and characteristic effect was a stimulation of respiration which usually set in after a short latency period and often lasted for as long as half an hour. This effect was observed following intracisternal as well as intraventricular injections of even small doses, suggesting a stimulating effect on the respiratory centres. This effect was especially well marked in unanaesthetized animals in which a needle had been inserted into the III:rd ventricle under local anaesthesia. Since a dose of 5 U. corresponds to only 2—3 μg of the highly purified preparations,

the activity is of the same order of magnitude as for instance that of acetylcholine.

Intrathecal control injections of Ringer solution containing preparations of Substance P inactivated by chymotrypsin resulted in no effects of any kind, thereby proving the specificity of the effect produced by the active substance.

It was observed that repeated intraventricular injections of Substance P, especially in larger doses, often caused smaller effects. On certain occasions only an inhibition was noted. These results suggest that Substance P in higher concentrations has an inhibitory action on central autonomic functions.

The effects of intraventricularly injected Substance P on the blood pressure were on the whole less consistent and marked. A primary transient fall in blood pressure was usually observed, followed in some cases by a long-lasting small rise. After intra-arterial injections towards the centres a progressive fall was observed in several cases.

No marked effects were observed on reflex or ganglionic transmission after intravenous administration of even large doses of Substance P. The carotid occlusion test showed no alteration of the blood pressure response, and the response of the nictitating membrane was only very slightly reduced if at all. It therefore appears less likely that Substance P is of general importance for autonomic peripheral ganglionic transmission processes, even if it occurs in sympathetic ganglia (PERNOW 1953 a).

The presence of Substance P in the hypothalamic region made it of interest to study whether or not intraventricular administration could elicit changes in the release of antidiuretic hormone. No indications of any action of this kind were observed, however.

In the experiments of FELDBERG and SHERWOOD (1954) a number of "autonomic" drugs were injected into the lateral ventricle of the unanaesthetized cat and a variety of symptoms were observed, including characteristic changes in behaviour. Substance P in the rabbit as well as in the cat, tended to inhibit spontaneity, especially after administration of large doses.

While these experiments have not given any detailed information as to the site or mode of action of Substance P when acting from the cerebrospinal fluid, they indicate that this polypeptide exerts a central neurotropic action which is of interest with respect to its occurrence in the C. N. S.

As to the type of effect of Substance P it bears some re-

semblance both to acetylcholine and nicotine although several differences have also been noted. As in the case of these drugs it appears that small doses stimulate while larger doses tend to inhibit nervous functions.

Summary.

Intracisternal or intraventricular injections of small doses of highly purified Substance P elicit stimulation of respiratory frequency and amplitude in cats and rabbits.

The effects on the blood pressure of injections of Substance P into the III:rd ventricle were usually small and variable.

No consistent effects of moderately large doses administered intravenously were observed on the carotid occlusion test or on the effect of preganglionic stimulation on the nictitating membrane.

Intraventricular injection of Substance P had no definite effect on diuresis.

Substance P injected into a lateral ventricle caused a variety of symptoms of which a general inhibition of spontaneity was the most marked.

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From the Department of Physiology, Karolinska Institutet, Stockholm.

Histochemical Demonstration of AChE Activity in Isolated Nerve Cells.

By

EZIO GIACOBINI.

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Introduction.

The distribution of acetylcholinesterase (AChE, specific cholinesterase) in nervous tissue has been studied during the last 20 years by estimating the activity of this enzyme in various parts of the nervous system. Most of our knowledge about the distribution of AChE is based on biochemical determinations of the AChE activity made on gross specimens of tissue (see *e. g.* COUTEAUX and NACHMANSOHN 1938—1940, GLICK 1937—1938, BRÜCKE 1937, BOELL and NACHMANSOHN 1940, CROXATTO, HUIDOBRO, SALVESTRINI, DONOSO, SANHUEZA, LUCO 1940, COUTEAUX 1942, MENDEL and RUDNEY 1944, BOELL and SHIH-CHANG SHEN 1944, SAWYER and HOLLINSHEAD 1945, AUGUSTINSSON 1948, WHITTAKER 1951, WOLFGRAM 1954). On the basis of these investigations conclusions can, however, not be drawn as to the localization of AChE in single cells.

Different histochemical methods have been developed in order to localize the site of the AChE activity in different types of cells (GOMORI 1948, KOELLE and FRIEDENWALD 1949, BARNETT and SELIGMAN 1951, HOLT and WITHERS 1952, RAVIN, ZACKS and SELIGMAN 1953, CREVIER and BÉLANGER 1955). KOELLE and FRIEDENWALD introduced a method for the localization of AChE in which a medium containing acetylthiocholine (AThCh), copper glycinate and copper thiocholine is used for the incubation of tissue sections.

Thiocholine liberated by enzymatic hydrolysis precipitates as copper mercaptide. Under the influence of $(NH_4)_2S$ this com-

pound changes into brown amorfous deposits of copper sulfide which serve as an indicator of the enzymatic activity at the specific site. This method seems to fulfill adequately the requirements of enzyme substrate specificity and has been applied by several authors for the demonstration of AChE activity in various tissues (KOELLE and FRIEDENWALD 1949—1950, KOELLE 1949—1955, COUTEAUX and TAXI 1952, GEREBTZOFF 1953, HEBB and SILVER 1953, HULL 1954, SAUER 1954).

In 1954 ZAJICEK et al. applied a modified thiocholine method on suspended cells from mammalian bone marrow. Hitherto the histochemical investigations on the nervous system have been performed on tissue sections only. Therefore the results of these investigations provide only information concerning the relative AChE activity in the different nerve cells visualized in the section.

In the present investigation, a modification of the thiocholine method has been applied on single nerve cells in order to study the distribution of the enzymatic activity within the cell body and along the neurite.

Furthermore, in order to get detailed information as to the localization of the enzyme, the substrate was directly microinjected in the different parts of the axon, and single parts of the neurone, isolated with the help of a micromanipulation technique, were also tested separately.

Material and methods.

Microdissection technique. Most experiments were performed on various nervous structures from frog; in some experiments nervous tissue from albino rats and cats was also used. After decapitation of the animal the spinal and autonomic ganglia, as well as different nerves, were quickly dissected out and put into Ringer's solution. A small fraction of the ganglion or of the nerve was teased apart on a coverslip with two sharp metal needles. A small fragment of the tissue was then placed in a moist oxygenated chamber. The microdissection was carried out at a temperature of 18—20° C. A binocular microscope was used and the dissection was performed under a magnification of 50 or 80 ×. When studying the spinal ganglion cells the capsule of the ganglion was removed and a group of about 30—60 cells with their processes was carefully separated. The separation of the single cells was performed partly with the aid of thin metal micro-needles, partly with Zeiss micromanipulators carrying glass-needles, 5—15 μ thick.

When isolating the motor horn cells from the spinal cord a small fraction of the cord was isolated in the following way. An incision was made through the white matter from the lateral aspect of the cord.

The white matter was pushed aside until the grey matter was reached and exposed. A small piece of the grey matter was then taken out and placed on the coverslip.

When dissecting single nerve fibers a short segment of the nerve was cut and a few fibers were carefully isolated. The fibers were inspected at high magnification in phase-contrast.

Since the nerve cells are rapidly damaged, the preparations were made as quickly as possible. The complete isolation was usually performed within 15—20 minutes. The cells and the nerve fibers which by microscopical inspection were found to be injured were discarded.

About 200 preparations were made. Since each preparation contained approximately 50 cells, about 10,000 nerve cells and fibres were examined.

Histochemical technique. For the demonstration of the enzyme activity a modification of Koelle's method was used (ZAJICÉK et al. 1954). The first step in this method is the same as in Koelle's method but neither is Na_2SO_4 used for the precipitation of the enzyme nor ammonium sulfide for the precipitation of copper sulfide. Thus, the thiocholine liberated by the enzymatic hydrolysis reacts with copper-glycinate, and the reaction product precipitates as insoluble needle-shaped crystals in the regions within which the enzyme is localized. These crystals have been recently identified as copperthiocholine sulfate (MALMGREN and SYLVÉN 1955).

Table I.

Substrate	Inhibitor	Types of enzyme activity demonstrated
Acetylthiocholine	None	All ChEs
»	DFP 10^{-7} M	AChE
»	Physostigmine 10^{-6} M	Control
Butyrylthiocholine	None	Non specific ChE
»	DFP 10^{-7} M	Control
»	Physostigmine 10^{-6} M	Control

Thus, the incubation medium used consisted of: 0.6 ml Cu glycine (0.002 M), 1.5 ml maleate buffer (pH 6.4); 0.6 ml MgC_2 (9.5 %), AThCh (0.004 M), 1.2 ml CuThCh to saturation. AThCh and BuThCh were used as substrates according to Table 1.

It is known that cells containing the AChE split AThCh at a high rate but not BuThCh. Non-specific cholinesterase hydrolyzes both substrates. The non-specific cholinesterase was inhibited by incubating with a solution of 10^{-7} M DFP (HAWKINS and MENDEL 1947, KOELLE 1950). It is also known that AChE is only slightly inhibited by DFP at this concentration. If a typical ChE inhibitor like physostigmine was added, the formation of crystals was totally prevented.

A drop of the incubation medium described was added to the isolated nerve cells or fibers. The cell suspension was covered with a cover slip and evaporation was prevented by surrounding the cover glass with a thin layer of vaseline. The minute crystalline deposits of copper-thiocholine sulfate indicating ChE activity were examined at different incubation times in the isolated cell under high magnification in phase-contrast microscopy and photographed.

Results.

A. Spinal ganglion cells.

Both in frog and rat the spinal ganglion cells are unipolar and about spherical in shape, and their size varies between 25 and 80 μ. The cytoplasm is granulated, has a high degree of viscosity and is relatively resistant to mechanical insults.

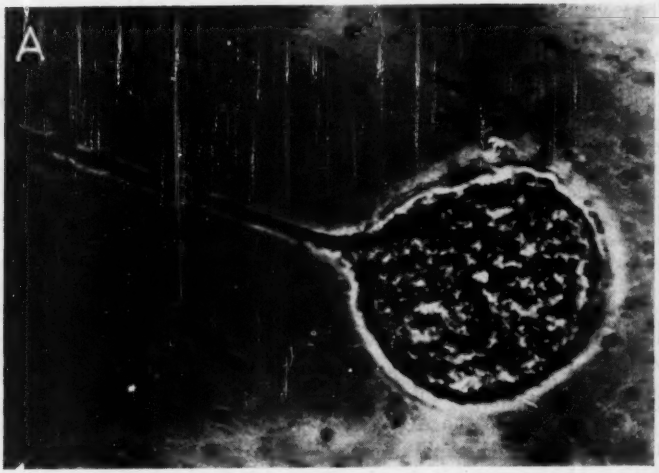
With respect to the AChE activity two types of cells could be distinguished in the spinal ganglia. About 85—90 % of the total cell population exhibited only slight AChE activity. If BuThCh was added, many of these cells showed significant concentrations of non-specific cholinesterase. About 10—15 % of the total number showed a very high concentration of AChE. In these cells the formation of crystals started as early as some minutes after the incubation (see Fig. 1 A). Small rod-shaped deposits were first observed which rapidly grew to needle-shaped crystals, about 2—3 μ long (Fig. 1 B).

The AChE activity was localized to the cytoplasm and the neurite but the nucleus did not show any activity. A high degree of AChE activity was often found in the region of the axon hillock and crystals were observed along the whole length of the axon (see Fig. 1 B and C). Capsular cells (rat) showed a high degree of non-specific activity.

No morphological difference could be observed between the cells which showed a high AChE activity and those with a slight or insignificant activity. In the ganglion the two types of cells were intermingled and did not show any special distribution. No crystals were formed if physostigmine (10^{-6} M) was added to the preparation.

B. Sympathetic ganglion cells.

The cells of the sympathetic ganglion of the frog were easier to isolate than those of the rat and the cat. In frogs the sympathetic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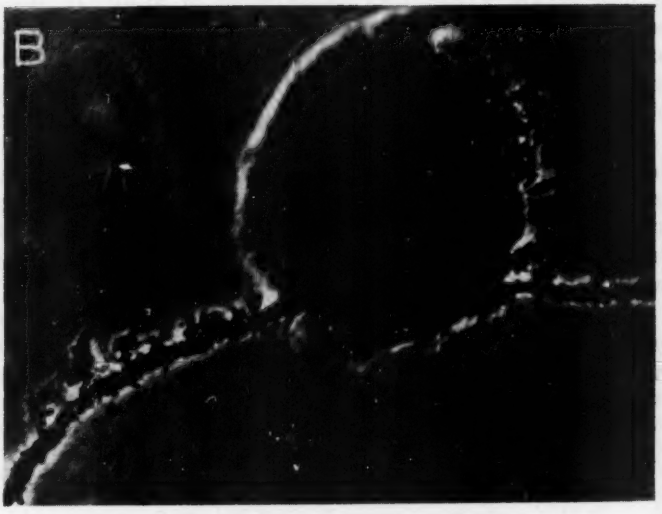

B

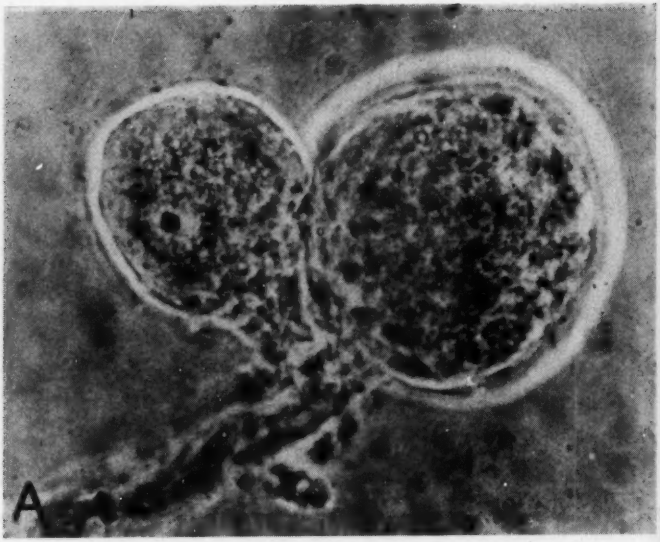
Fig. 1. Spinal ganglion cells from rat. The AChE activity after a short incubation (3—5 min.) demonstrated by small granular deposits of Cu-thiocholine sulfate (A). If the incubation time is prolonged to 15 min. these formations become rod- and needle-shaped crystals covering all the cytoplasm and the neurite (B). The neurite of the bipolar cell in B is shown at higher magnification in C. Note the presence of numerous crystals inside and around the axon. Magnification in A and B 320 and in C 800 ×.

ganglion cells are round or oval and usually unipolar. In mammalians these cells are usually multipolar and provided with dendrites.

On the basis of the AChE activity the sympathetic ganglion cells could be divided into three groups. About 40—50 % of the total number of cells showed a slight or no AChE activity (Fig. 2 A). Another group of cells showed a moderate AChE activity. The degree of activity and the relative number of these cells was difficult to determine with histochemical methods. Approximately 50 % of the total number of cells were considered to belong to this group.

A certain number of the cells belonging to these two groups showed a moderate activity when BuThCh was used as substrate in the incubation medium. The distribution of non-specific ChE was different in three types of animals. The ChE-bearing cells were more numerous in the frog than in the rat and the cat.

Finally, about 5—10 % of the total number of ganglion cells showed a high AChE activity (Fig. 2 B and C). After an incuba-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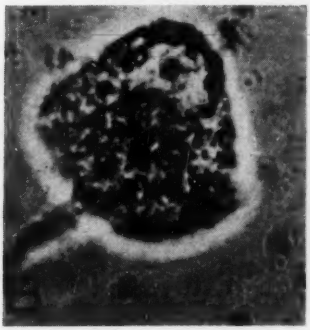

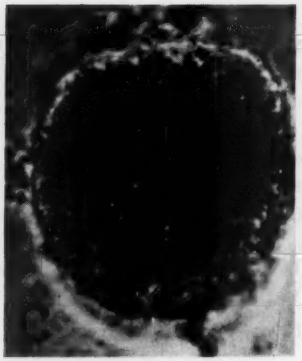

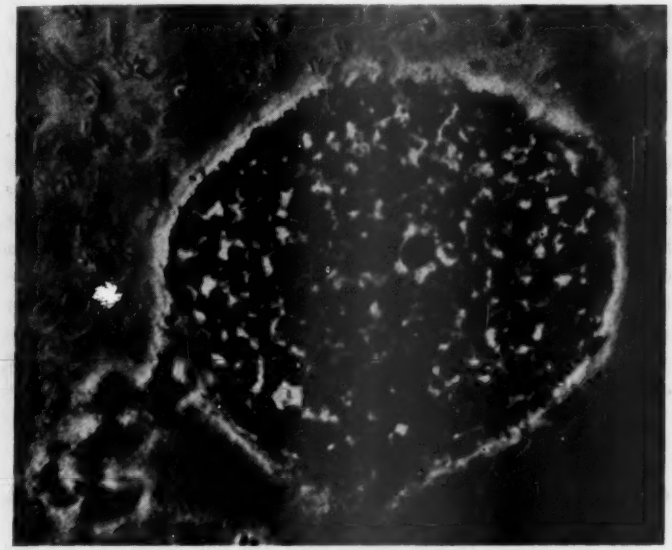

Fig. 2. AChE activity in sympathetic ganglion cells from frog. A, absence of crystal formation after 60 min. incubation. (Magnification 320 ×.) B, and C, abundant granular deposits after 3—5 min. incubation in the cytoplasm of two neurones belonging to the same preparation, demonstrates a high AChE activity. (Magnification 160 ×.) D, early formation of small granular deposits after 5 min. incubation (note the marked layer inside the cell membrane). (Magnification 800 ×.)

tion time of 3—5 minutes small crystals were noted in the cytoplasm of these cells. About 30 minutes later the whole cytoplasm was filled with small crystals lying so close to each other that the structural details of the cell could not be seen. The relative amount of elements showing a high AChE activity was less numerous in the cat and the rat than in the frog.

An early formation of small rod-shaped crystals in a marked layer surrounding the cell membrane (see Fig. 2 D) could be seen in some of the cells which showed a high or moderate AChE activity.

The nuclei of the sympathetic ganglion cells do not seem to have any significant activity (see Fig. 2 B).

Most of the medullated preganglionic fibers entering the ganglia through the white ramus showed a strong AChE activity. It has been demonstrated (Dogiel 1895, Huber 1899, Ranson and

BILLINGSLEY 1918, DE CASTRO 1923—1932, HILLARP 1946) that these fibers divide inside the ganglion into two or three medullated fibers branching into non-medullated collaterals. These intraganglionic fibers also showed a very high AChE activity.

The postganglionic sympathetic fibers, most of which are unmyelinated or provided with a thin myelin sheath, did not show any significant formation of crystals during the first hour after incubation. Only a limited number of fibers, probably those belonging to the cell bodies with a high AChE activity, showed a certain degree of activity already after some minutes of incubation. Capsular glial cells showed a strong positive reaction for non-specific AChE.

No morphological differences could be found between the three different types of cells.

C. Cells from the anterior and lateral horns of the spinal cord.

The cell bodies in the spinal cord are not protected by any capsule and the cytoplasm of the spinal cells is very fragile.

When ventral and lateral horn cells were incubated in a medium containing AThCh, the appearance of crystals inside the cytoplasm and the axon (5 minutes after the incubation) demonstrated a very high AChE activity. About 30 minutes after the incubation the cytoplasm of these cells was completely filled with small crystals. At the same time crystals also appeared in the dendrites. Neuroglial cells (protoplasmic astrocytes) belonging to the same segment showed a high concentration of non-specific ChE.

When these cells were first incubated (30 minutes) in a solution containing DFP (10^{-7} M at pH 6.4) and then incubated in a medium with AThCh, the formation of crystals was only slightly inhibited, demonstrating the presence of AChE.

D. Anterior and posterior roots.

The majority of the fibers isolated from the *anterior* roots was highly active (Fig. 3 A) and 15—20 % of the total number showed only a slight or no formation of crystals after incubation during 2 hours. Most of the fibers obtained from the *posterior* roots showed

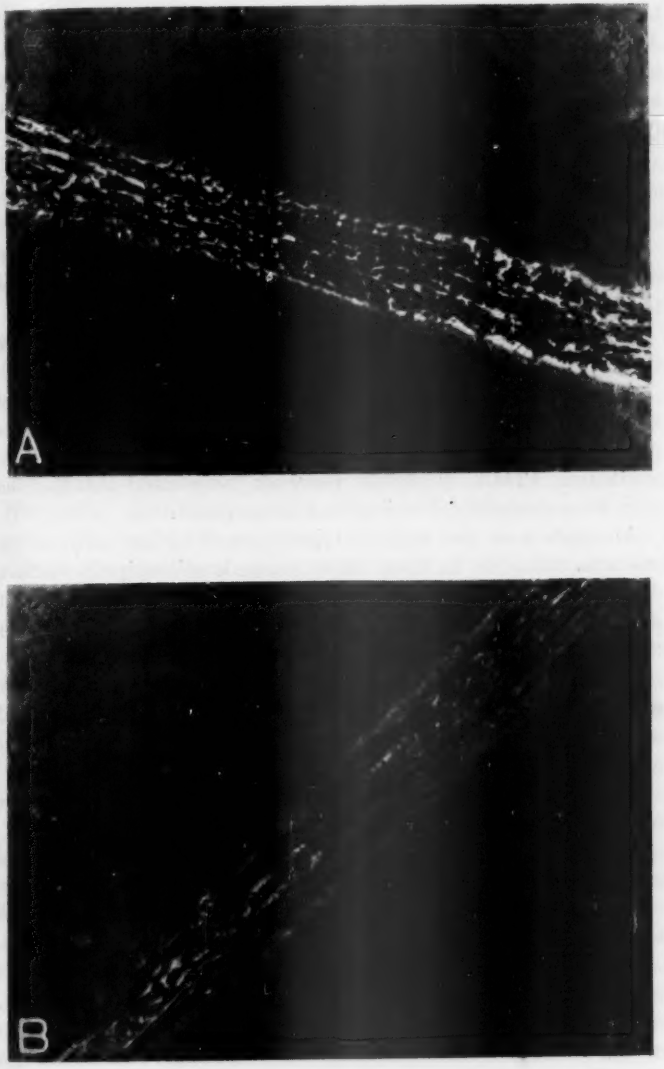

Fig. 3. A, a group of four fibers belonging to an anterior root (rat) showing formation of crystals. Incubation time 15 min. (Magnification 160 ×.) B, slight formation of small rod-shaped crystals within and around a fiber isolated from a posterior root (rat). Incubation time 60 min. (Magnification 800 ×.)

but a slight reaction when AThCh was used as substrate (Fig. 3 B). About 20 % showed a clear deposition of crystals after 90 minutes' incubation. Some of these fibers showed a non-specific activity high enough to give rise to crystal formation 45 minutes after the incubation.

E. Auxiliary microsurgical experiments.

Some additional experiments were performed in order to obtain information on the localization of the enzyme in different parts of the neurone.

1. *Nerve fiber preparations.* In these experiments isolated fibers from the frog's sciatic nerves were used.

Attempts were made to incubate segments of the axon from which the myelin sheath had been taken off. In such preparations no crystals appeared, when they were incubated in a medium containing AThCh. It should, however, be pointed out that the cylinder axis rapidly deteriorates when exposed.

Attempts were also made to inject the incubation medium into the axis cylinder. In these experiments a micropipette with a diameter of 1 to 3 μ was introduced into the fiber at different depths. When the injection was made just inside the axis cylinder the solution spread to the surrounding portions of the fiber. Formation of crystals were detected after a period of time which was generally shorter than when isolated fibers were incubated in the same medium with the usual technique. Crystals always appeared both around and inside the fiber.

Attempts to determinate the difference in activity between the nodal and the internodal segments failed.

Experiments were also made on neurites in which a wound was made by introducing a thin glass micro-needle. In fibers the myelin sheath of which was wounded at regular intervals the crystal formation seemed to be particularly high at the wounded points.

2. *Cell body preparations.* Spinal and sympathetic ganglion cells of frog and rat were found most suitable for these experiments.

When isolated parts of the cytoplasm were suspended in a drop of the incubation medium a crystal formation was noted. No crystals were observed in the surrounding medium.

Positive tests were found in isolated parts of the cytoplasm having a surface of about 15—20 μ^2.

Isolated nuclei never exhibited any formation of crystals.

Discussion and Conclusions.

Although the histochemical methods used for demonstration of AChE in tissue sections have proved to be useful in studying the relative enzyme activity in the different neurones visualized in the sections, they are subject to several unfavourable conditions.

Among the different factors limiting the use of the thiocholine method of Koelle on tissue sections for the study of the distribution of the AChE in different parts of a neurone should be mentioned:

a) that the diffusion of the initial product of the hydrolysis (Cu.thiocholine) or of the secondary product of the reaction (Cu.sulphide) is considerable in presence of structures having a high content of enzyme; diffusion and secondary precipitation at a contiguous site make it very difficult to assert whether or not the area in which the precipitation product is seen represents the actual localization of the enzyme;

b) that the fixation of the tissue in different mediums substances (formaline, alcohol, dioxane, acetone) alters or destroys the enzyme activity; the fixation may also give rise to artifacts which disturb the exact localization of the product of the histochemical reaction;

c) that the thickness of the sections and the irregular background staining due to diffusion make it difficult to localize exactly the product of the reaction in the cellular structures;

d) that the sectioning of the tissue may facilitate diffusion of the enzyme inside the cell for example from the cytoplasm to the nuclear membrane;

e) that the second step of the histochemical reaction (conversion of AThCh into CuS) partly depends upon the amount of CuThCh which has been formed during incubation; if this amount is large, the heavy deposits of CuS within large areas of the preparation obscure the structural details.

By using the technique applied in the present investigation these sources of error are partly eliminated. Since the nervous elements in each preparation are few and separated from each other the diffusion phenomena are of minor importance and can be controlled. Artifacts and inactivation of the enzyme due to fixation, imbedding and sectioning are avoided.

Furthermore, it is easier to localize the deposits of the reaction product in single nerve cell preparations than in tissue sections in which different components lay closely bundled to each other.

For reasons mentioned above the second step of the histochemical reaction of Koelle was omitted, the formation of the Cu.thiocholine sulfate crystals instead being followed microscopically by inspection.

In neurones belonging to the spinal, sympathetic and parasympathetic ganglions and in some of the cells in the anterior and lateral horns of the spinal cord, large concentrations of AChE could be demonstrated in the cytoplasms and neurites. The deposition of the crystals showed that in these different types of cells there was a high concentration of AChE in the cytoplasm, at axon hillock and at the surface of the axon, whereas the nuclei as well as the membranes of the cell bodies did not show any activity. The attempts to evaluate the degree of AChE activity in the different parts of the axon (root, node, internode) did not give any valid results.

When small fractions of the cytoplasm were suspended in a drop of the incubation medium, formation of crystals was observed after a short incubation time. Positive tests were obtained on isolated parts of the cytoplasm having a surface of about 15—20 μ^2. No crystal formation was detected in the surrounding medium. These findings indicate that there may be a binding of the enzyme molecule to the cytoplasmic structures. The co-existence of the enzyme in the cell-body and the axon seems to characterize the AChE bearing neurones. However, in order to elucidate these problems a method for quantitative estimation of AChE activity in single nerve cells has been applied and the preliminary results of these investigations have recently been published (GIACOBINI and ZAJICEK 1956).

According to the results of the present investigations, about 10—15 % of the cells in the spinal ganglions exhibited a high specific activity and also in the sympathetic ganglions about 60 % of the cells showed a pronounced AChE activity. Comparing these results with those reported by KOELLE (1951—1955) employing the same substrate and with those obtained by RAVIN, ZACKS and SELIGMAN (1953) employing β-naphtylacetate it should be noted that the relative amount of AChE bearing cells in the spinal and sympathetic ganglia of rat and cat was found to be considerably larger than reported by these authors.

Some discrepancies were also found with respect to the site of AChE activity previously demonstrated in ganglion cells. It should, however, be pointed out that no definitive conclusions

can yet be drawn as to the localization of the enzyme by means of the results provided by histochemical methods only, and that more sensitive techniques must be developed for a more accurate determination of it in the single parts of the neurone.

Summary.

A modification of the thiocholine method of Koelle (1951) has been used for the demonstration of the AChE activity in single nerve cells isolated from the surrounding tissue by means of microdissection technique.

In neurons in the spinal, sympathetic and parasympathetic ganglions, as well as in the anterior and lateral horns of the spinal cord, large concentrations of AChE could be demonstrated inside the cytoplasm and neurite after a very short incubation time. By using this method the relative amount of such AChE bearing cells was found to be considerably higher than in previous investigations.

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